CONTENTS

No. 1 JANUARY, 1948

JOSEF WARKANY AND CAROLYN B. ROTH. Congenital malformations induced in rats by maternal vitamin A deficiency. II. Effect of varying the preparatory diet upon the yield of abnormal young. Four figures 1

LORRAINE S. GALL, PAUL F. FENTON AND GEORGE R. COWGILL. The nutrition of the mouse. II. Effect of diet on the bacterial flora of the intestine and the cecum 13

LORRAINE S. GALL, BARBARA A. ILLINGWORTH, GEORGE R. COWGILL AND PAUL F. FENTON. The nutrition of the mouse. III. Relation of diet to the synthetic activity of the predominating flora isolated from the small intestine and cecum 27

E. D. SMITH, B. H. ERSHOFF, R. J. WINZLER AND H. J. DEUEL, JR. Effects of B vitamins, liver and yeast on growth under cold room and room temperature conditions 39

ANTON J. CARLSON AND FREDERICK HOELZEL. Effect of vegetarian self-selection diets on reproduction and the growth of offspring of rats 49

J. R. COUCH, W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN. Relation of carbohydrate to intestinal synthesis of biotin and hatchability in mature fowl. Five figures 57

MARTHA E. HOLLINGER. Human utilization of ascorbic acid from mustard greens. Two figures 73

C. E. FRENCH, A. BLACK AND R. W. SWIFT. Further experiments on the relation of fat to economy of food utilization. III. Low protein intake .. 83

B. S. SCHWEIGERT, H. L. GERMAN, P. B. PEARSON AND R. M. SHERWOOD. Effect of the pteroylglutamic acid intake on the performance of turkeys and chickens 89

RUTH MAJOR AND BETTY M. WATTS. The relation of fed and injected tocopherols to development of rancidity in the stored meat and utilization of carotene by the rabbit 103

THEODORE E. FRIEDEMANN, VELMA M. KINNEY, GEORGE H. BERRYMAN, CHARLES R. HENDERSON AND JOHN B. YOUMANS. The effect of dietary restriction of B-complex vitamins and protein on the excretion of creatinine by human subjects. Four figures 117

No. 2 FEBRUARY, 1948

B. CONNOR JOHNSON, T. S. HAMILTON, W. B. NEVENS AND L. E. BOLEY. With the technical assistance of K. A. Burke and J. A. Pinkos. Thiamine deficiency in the calf. Three figures 137

A. E. SCHAEFER, C. K. WHITEHAIR AND C. A. ELVEHJEM. Unidentified factors essential for growth and hemoglobin production in foxes. Two figures .. 147

A. E. SCHAEFER, S. B. TOVE, C. K. WHITEHAIR AND C. A. ELVEHJEM. The requirement of unidentified factors for mink. One figure 157

RALPH HOAGLAND, N. R. ELLIS, O. G. HANKINS AND G. G. SNIDER. Supplemental value of certain amino acids for beef protein 167

ANTHONY A. ALBANESE, L. E. HOLT, JR., VIRGINIA I. DAVIS, SELMA E. SNYDERMAN, MARILYN LEIN AND EMILIE M. SMETAK. The isoleucine requirement of the infant. One figure 177

ROBERT R. SPITZER, GEORGE MARUYAMA, L. MICHAUD AND PAUL H. PHILLIPS. The role of vitamin D in the utilization of phytin phosphorus 185

M. B. GILLIS, L. C. NORRIS AND G. F. HEUSER. The utilization by the chick of phosphorus from different sources 195

GLADYS EVERSON, ELIZABETH WHEELER, HELEN WALKER AND W. J. CAULFIELD. Availability of riboflavin of ice cream, peas, and almonds judged by urinary excretion of the vitamin by women subjects 209

ALBERT E. SOBEL, MARVIN SHERMAN, JACQUELINE LICHTBLAU, SELIG SNOW AND BENJAMIN KRAMER. Comparison of vitamin A liver storage following administration of vitamin A in oily and aqueous media 225

GRACE H. BELL, BRADLEY T. SCHEER AND H. J. DEUEL, JR. Niacin excretion in the rat in relation to tryptophane, pyridoxine, and protein content of the diet 239

G. M. WARD AND J. T. REID. Mineral metabolism studies in dairy cattle. II. Effect of calcium and manganese and other trace elements on the metabolism of lipids during early lactation 249

HAZEL C. MURRAY. The biological value of the protein of field pea products with a comparison of several methods used for this determination 257

BENJAMIN H. ERSHOFF. The effects of B vitamins, liver and yeast on atabrine toxicity in the rat 269

E. M. SCOTT, SARAH JANE SMITH AND ETHEL L. VERNEY. Self selection of diet. VII. The effect of age and pregnancy on selection. Two figures .. 281

No. 3 MARCH, 1948

ROBERT A. HARTE, JOHN J. TRAVERS AND PETER SARICH. The effect on rat growth of alternated protein intakes. One figure 287

HARRY J. DEUEL, JR., AMBER L. S. CHENG AND MARGARET G. MOREHOUSE. The digestibility of rape-seed oil in the rat 295

HARRY J. DEUEL, JR., SAMUEL M. GREENBERG, EVELYN E. STRAUB, DOROTHY JUE, CHESTER M. GOODING AND CARL F. BROWN. Studies on the comparative nutritive value of fats. X. On the reputed growth-promoting activity of vaccenic acid 301

F. H. KRATZER AND DELBERT WILLIAMS. The glycine requirement of young poults. One figure 315

WALTER, C. RUSSELL, ARTHUR E. TEERI AND KLAUS UNNA. Growth and reproduction of swine on a purified diet. One figure 321

VICTOR A. DRILL AND TED. A. LOOMIS. Production of functional and fatty changes in the liver by a chronic vitamin B complex deficiency, and interrelationships with protein intake. Fourteen figures 333

M. B. GILLIS, G. F. HEUSER AND L. C. NORRIS. Pantothenic acid in the nutrition of the hen. Two figures 351

J. Milton Bell. An adjustable cylindrical cage for use in metabolism studies with young pigs. Two figures 365

E. Potts Anderson, J. Knox Smith, C. A. Elvehjem and Paul H. Phillips. Dental caries in the cotton rat. X. The effect of fluidity of the ration 371

R. J. Westfall and S. M. Hauge. The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. Two figures 379

M. C. Blanchaer and Eileen Cameron. The relation of thiamine clearance to blood levels in man. One figure 391

No. 4 APRIL, 1948

D. M. Hegsted. The determination of minimum vitamin requirements for growth. Three figures 399

D. M. Hegsted and Richard L. Perry. Nutritional studies with the duck. V. Riboflavin and pantothenic acid requirements. One figure 411

Eva Hartzler. False high values for ascorbic acid in guava juice. A note on the use of the colorimetric method with 2,4-dinitrophenylhydrazine .. 419

Elfriede F. Brown and Agnes Fay Morgan. The effect of vitamin A deficiency upon the nitrogen metabolism of the rat. Two figures 425

Elfriede F. Brown and Agnes Fay Morgan. Nitrogen metabolism of the normal and the vitamin A-deficient rat as affected by thyroid administration. One figure 439

Frances A. Johnston, Ruth Frenchman and Euphemia Deade Boroughs. The absorption of iron from beef by women 453

J. F. Sykes, T. R. Wrenn and S. R. Hall. The effect of inanition on mammary-gland development and lactation. Two figures 467

Robert John Evans and James McGinnis. Cystine and methionine metabolism by chicks receiving raw or autoclaved soybean oil meal 477

Winfrey Wynn and John Haldi. The erosive action of various fruit juices on the lower molar teeth of the albino rat 489

S. H. Rubin, J. M. Cooperman, M. E. Moore and J. Scheiner. The physiological availability of pantothenyl alcohol. One figure 499

Clara M. Taylor, Mina W. Lamb, Mary E. Robertson and Grace MacLeod. The energy expenditure for quiet play and cycling of boys seven to fourteen years of age. One figure 511

No. 5 MAY, 1948

Jean Mayer and W. A. Krehl. The relation of diet composition and vitamin C to vitamin A deficiency. One figure 523

Roy E. Butler and Herbert P. Sarett. The effect of isocaloric substitution of alcohol for dietary carbohydrate upon the excretion of B vitamins in man 539

Eugene M. Sporn and C. A. Elvehjem. Growth and reproduction of rats fed army combat rations 549

Eugene M. Sporn, W. R. Ruegamer and C. A. Elvehjem. Studies with monkeys fed army combat rations. Two figures 559

Orlando Olcese, P. B. Pearson and B. S. Schweigert. The synthesis of certain B vitamins by the rabbit 577

J. Thomas Reid and George M. Ward. Mineral metabolism studies in dairy cattle. III. Manganese metabolism in the lactating bovine. One figure 591

W. D. Armstrong. With the technical assistance of Mary Gouze and Marcy Peterson. Influence of nutritional factors on skeletal atrophy from disuse and on normal bones of mature rats 597

Haydee R. Estremera and W. D. Armstrong. Effect of protein intake on the bones of mature rats 611

Mary Elizabeth Reid. Urinary excretion of ascorbic acid by guinea pigs at different ages. Two figures 619

No. 6 JUNE, 1948

Charles F. Bassett, J. K. Loosli and Ford Wilke. The vitamin A requirement for growth of foxes and minks as influenced by ascorbic acid and potatoes 629

D. Breese Jones, Alvin Caldwell and Katherine D. Widness. Comparative growth-promoting values of the proteins of cereal grains 639

Carl A. Kuether and Victor C. Myers. The nutritive value of cereal proteins in human subjects 651

Philip Handler and Richard H. Follis, Jr. The role of thyroid activity in the pathogenesis of hepatic lesions due to choline and cystine deficiency. Two figures. 669

Irene T. Greenhut, R. J. Sirny and C. A. Elvehjem. The lysine, methionine and threonine content of meats 689

R. M. Johnson and C. A. Baumann. Relative significance of growth and metabolic rate upon the utilization of vitamin A by the rat 703

Flowayne Holt and Florence I. Scoular. Iron and copper metabolism of young women on self-selected diets 717

Lillian Belle Cosner and Cecilia Schuck. Suitability of the Bourquin-Sherman diet for riboflavin assays and results obtained in the assay of evaporated milk. Two figures 725

American Institute of Nutrition. Proceedings of the twelfth annual meeting, Atlantic City, New Jersey, March 14–19, 1948 733

CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL VITAMIN A DEFICIENCY

II. EFFECT OF VARYING THE PREPARATORY DIET UPON THE YIELD OF ABNORMAL YOUNG [1]

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FOUR FIGURES

(Received for publication August 12, 1947)

Congenital anomalies in the offspring of vitamin A deficient animals have been described repeatedly. The literature on this subject has been reviewed by Warkany and Schraffenberger ('46). The studies of Hale ('33, '35, '37) are of great importance, since genetic factors could be ruled out as the cause of the malformations obtained. Hale fed gilts of known stocks a diet deficient in vitamin A and possibly in other nutritional factors. The offspring showed anophthalmos or microphthalmos, accessory ears, harelip and cleft palate, subcutaneous cysts and misplaced kidneys. For experimentation on a large scale the production of congenital malformations by maternal vitamin A deficiency in a small laboratory animal appeared desirable. Anderson ('41) reported briefly the occurrence of diaphragmatic hernia in about 25% of the young of rats bred on a diet containing the minimum amount of vitamin A required for pregnancy and parturition. A small number of the controls which were fed halibut liver oil, showed the same lesion.

[1] This work was aided in part by a grant from the Nutrition Foundation, Inc., New York.

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Warkany and Schraffenberger ('44, '46) reported congenital anomalies of the eyes in the offspring of rats that had been depleted of vitamin A. Female rats were raised on a preparatory diet (diet U) which contained small measured amounts of carotene. The carotene supplement was necessary to make possible growth and maturation of the females; however, it was insufficient to provide vitamin A for storage. When the female rats reached a weight of 150 to 160 gm and had regular oestrous cycles they were given a purified diet completely free of vitamin A and carotene (diet W). While on this diet the females were bred and kept throughout pregnancy. Under such dietary conditions few animals carry their young to term. Many females become sterile; some stop having oestrous cycles; others mate but resorb their young. In 1 series of experiments (Warkany and Schraffenberger, '46) only 7 of 140 females carried their offspring to term and 39 young were obtained. All the eyes of the young that were serially sectioned were found to be abnormal. The most constant abnormal finding was a fibrous retrolenticular membrane in place of the vitreous body (fig. 1B-H). In addition there were frequently colobomas, eversion, abnormal structure and folding of the retina, rudimentary development of the iris and of the ocular chambers, defects of the cornea and of the conjunctival sac, and lack of fusion of the lids. In the last case the abnormal eyes could be recognized by external inspection ("open eyes") (fig. 2). These experiments on rats were repeated with slight modifications by Jackson and Kinsey ('46) who confirmed the findings of Warkany and Schraffenberger. The study of Jackson and Kinsey was supplemented by determinations of the vitamin A level of pregnant rats which was found to be less than 12 I.U. per 100 ml in all the mothers who produced young with abnormal eyes. In the series of these authors only 4 of 57 females subjected to the dietary conditions described carried their fetuses to advanced stages of development, and Jackson and Kinsey concluded "that the ocular defects occur in the young rat only when the maternal vitamin A deficiency is extremely severe, so ad-

vanced, in fact, that fetal resorption is common and normal birth is impossible."

Since the rate of fertility was very low in the original experiments and the yield of abnormal offspring very small, improvements of the experimental methods were attempted by modifications of the preparatory diet. These modifications are reported in the present communication. In the previous experiments rats of the Sprague-Dawley strain only were used. It seemed advisable to repeat the experiments with rats from a different source and in this series a large number of rats obtained from the Albino Farms, Red Bank, N. J. were included.

METHODS

Female rats weighing 30 to 40 gm were placed on the preparatory diet (diet U) which had the following percentage composition: Ground whole wheat, 74; crude casein,[2] 15; brewers' yeast,[3] 10; sodium chloride of C.P. grade, 1. According to the supplements added to this diet the animals were divided into 4 groups. One hundred and sixty-five females were given a supplement of only 4 μg of carotene every tenth day (group I). Since this supplement proved insufficient for growth and maturation, each rat received in addition approximately 2 gm of frozen horsemeat daily. This group was kept on diet U until mating occurred, at which time the females weighed between 150 and 160 gm. Then the females were placed on diet W. Group II, consisting of 34 females, received a supplement of 12 μg carotene every tenth day. In group III 33 animals were given 25 μg carotene every tenth day. A fourth group consisting of 28 females received during the first month supplements of 25 μg of carotene every tenth day. In the following month a loss of weight was observed and therefore the carotene intake was doubled during the last 2½ weeks of the preparatory period. Groups II, III and IV were placed on diet W upon reaching maturity and attempts to breed them were made after the change to this purified diet. In these 3 groups mating took place usually within 1 week or 10 days after the

[2] Borden. [3] Mead Johnson.

change to diet W; but even after 4 weeks on the purified diet mating occasionally occurred. Diet W, a purified diet completely free of vitamin A and carotene, had the following percentage composition: Sucrose, 68; vitamin test casein, 18; vegetable oil, 10; salt mixture of Hubbell, Mendel and Wakeman ('37), 4. To 100 gm of this diet was added 0.8 μg thiamine hydrochloride; 0.8 μg pyridoxine hydrochloride; 0.8 μg riboflavin; 1 μg calcium pantothenate; 10 μg niacinamide; and 100 μg choline chloride. This diet was supplemented every tenth day by vitamin D (1 drop of a 1:4 dilution of drisdol with olive oil), vitamin E (1 drop of a solution of 5 gm alpha-tocopherol in 100 ml of olive oil), and vitamin K (1 drop of a solution of 1 μg of 2-methylnaphthoquinone in 5 ml of olive oil). On this diet the female rats were bred and kept throughout pregnancy. Vaginal smears were made daily and the weight curves were closely observed. This made possible the recognition of the phase of oestrus; the beginning of pregnancy (by finding sperm); the continuation of pregnancy; or beginning of resorption (by finding of large amounts of blood in the vaginal smear and by a marked loss of weight). The day on which sperm were found was counted as the first day of pregnancy. The profuse bleeding of resorption can be distinguished as a rule from the normal blood sign, which appears between the tenth and the fifteenth days of gestation and which consists of a small amount of blood in the vaginal smear. When early termination of pregnancy threatened, the animal was opened and the fetuses removed. When the young were carried to term the mothers were opened on the twenty-second day of gestation. Many young thus removed were alive but none could be raised. The mothers were sacrificed and therefore no second litters were obtained. The eyes of at least 1 animal in each litter were sectioned serially. If no retrolenticular membrane was found the animal was considered normal but if a membrane was seen in any section the animal was considered abnormal. When the eyes of 1 young in a litter were found to be abnormal the entire litter was considered and counted as abnormal. This method of classification appeared

to be justified, since in 14 litters selected at random the eyes of all the littermates when serially sectioned were found to be so similar that all the members of each litter could be considered normal or abnormal (fig. 1). There was also marked external resemblance between the animals of the same litters (fig. 2).

RESULTS

Of 260 females reared on the preparatory diet U, 22 had cycles but did not mate. One hundred and twenty-four females mated but did not have any issue. Litters were obtained from 114 females but only 30 of these litters were carried to term (table 1). The rest were obtained prematurely and consisted of fetuses of various gestational ages (table 2). Of the females used in this series 156 were of the Sprague-Dawley strain and 104 were obtained from the Albino Farms.

According to the presence or absence of a retrolenticular membrane in the eyes of 1 or of several members of a litter, 89 litters were considered abnormal and 25 normal. These litters consisted of a total of 820 young, 612 of which were considered abnormal and 208 normal. In addition to the retrolenticular membrane, other ocular defects resembling those described in our previous publication ('46) were seen in many of the eyes sectioned. In most eyes with a retrolenticular membrane the vitreous humour is absent and fibrous tissue occupies the entire space between the lens and the retina. However, in some of the abnormal eyes rudiments of the vitreous body are present and are transversed by a thick strand of fibrous tissue which starts at the head of the optic nerve and continues to the posterior surface of the lens (fig. 1). Many litters show external changes which are not observed in control animals. In figure 2 an abnormal litter of 7 young is compared to a normal newborn animal. The abnormals are smaller than the normal animal although they were removed from the mother's uterus on the twenty-second day of gestation. Owing to the edematous swelling of the subcutaneous tissue the narrowing at the neck is markedly reduced and the entire back presents a single convexity. All the animals of

this litter have "open eyes" which are best seen in specimens B and D. Subcutaneous hemorrhages are seen in C and D. The fingers and toes are well-developed but the dorsa of the

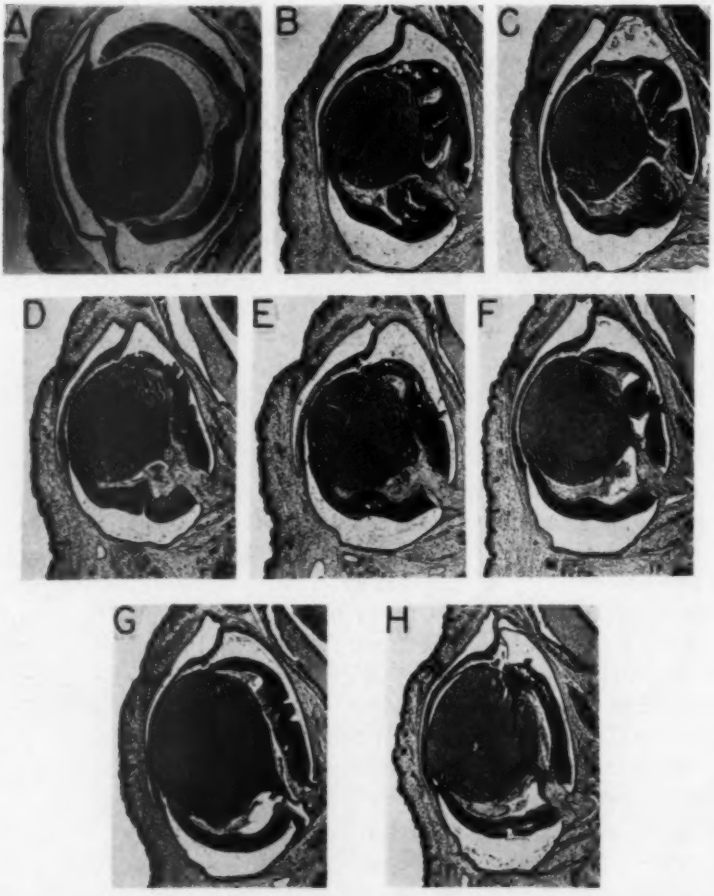

Fig. 1 Sections through eyes of newborn rats. A, control; B-H, offspring of vitamin A deficient mother. These sections represent the eyes of 7 littermates. The retrolenticular membrane is seen in every section, while only rudiments of a corpus vitreum are developed. A slight eversion of the retina is seen in section G only. (× 18).

hands and feet are puffy. The soles of the feet are turned inward which represents a retention of the fetal position.

In many young with abnormal eyes serial sections of the trunk and abdomen revealed various congenital anomalies of

Fig. 2 A, newborn rat, control; B-H, offspring of vitamin A deficient mother, removed from the uterus on the twenty-second day of gestation. These young, all littermates, show edema and inward rotation of the feet. The "open eye" can be seen in B and D, a cutaneous hemorrhage in D.

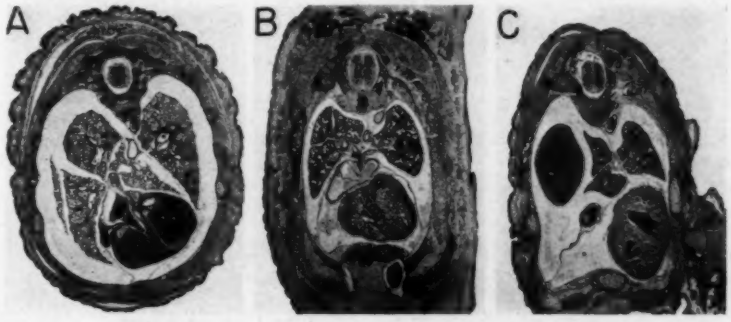

Fig. 3 Sections through the chest of newborn rats. A, control (× 3½); B and C (× 5½), offspring of vitamin A deficient mothers. In B the lung, the pleura and the heart muscle show retarded development. In C a lobe of the liver is seen in the right pleural cavity (diaphragmatic hernia).

the soft tissues. The lungs are underdeveloped and frequently retain their fetal position behind the heart and fail to grow forward. The pleural chambers border the pericardial cavity posteriorly and laterally but do not surround it (fig. 3 B and C). The heart muscle retains the spongy structure of the fetal

heart (fig. 3 B and C). In a number of specimens the right dorsolateral portion of the diaphragm is not developed and a lobe of the liver may protrude into the pleural space. This represents a diaphragmatic hernia (fig. 3 C). The kidneys are hypoplastic and the renal pelvis and the ureters are not distended as in the normal rat at the end of the gestational period. The space between the kidneys is narrow and fusion may take place in the lower poles, thus forming a horseshoe kidney. The testes are frequently undescended and may be found at the level of the kidneys or above (fig. 4 C). A detailed study

Fig. 4 Sections through the abdomen. A, control (× 3½); B and C, offspring of vitamin A deficient mothers. In B the renal pelvis seen in the left kidney is not unfolded (× 5). In C (20-day fetus; × 7) a fusion of the lower poles of the kidneys is seen (horseshoe kidney).

of these anomalies is being made at the present time and will be reported elsewhere. There is a tendency for hemorrhages to occur in the skin, the eyes and other organs. Keratinizing metaplasia was found by Wilson and Warkany ('47) in many abnormal young in the urethra distal to the termination of the genital ducts. In other parts of the body no abnormal keratinization was observed.

In all 4 groups abnormalities of the same type were obtained and there was no difference between the abnormalities in the offspring of rats of the Sprague-Dawley strain and those obtained from the Albino Farms. However, there were differences in the incidence of abnormalities and in the fer-

tility rates in the 4 groups according to the different amounts of carotene in the preparatory period.

Groups I and II had the lowest fertility rate and the highest incidence of abnormal young; group IV had the highest fertility rate and the lowest incidence of abnormal young.

Table 2 shows that most of the younger fetuses that were dying in utero or were threatened by intrauterine death were

TABLE 1

Breeding results with females raised on the preparatory diet U with various supplements and bred on diet W.

CATEGORY OF INTEREST	TOTAL	SUPPLEMENTS OF PREPARATORY DIET			
		Group I: 4 μg carotene [1] (and horsemeat)	Group II: 12 μg carotene [1]	Group III: 25 μg carotene [1]	Group IV: 25–50 μg carotene [1]
Number of females	260	165	34	33	28
Cycles but no matings	22	17	3	2	0
Matings but no issue	124	88	19	13	4
Number of litters	114	60	12	18	24
abnormal	89	52	12	12	13
normal	25	8	0	6	11
Number of young	820	428	76	139	177
abnormal [2]	612	363	76	96	77
normal [2]	208	65	0	43	100

[1] The carotene supplements were given every tenth day. The animals in group I received 2 gm frozen horsemeat daily.

[2] These figures are based on the assumption that the entire litter was abnormal, when anomalies were found in the eyes of at least 1 member of the litter (see page 4).

TABLE 2

Gestational age of 114 litters and of 820 young.

DAY OF PREGNANCY	15th	16th	17th	18th	19th	20th	21st	22nd
Total number of litters	3	6	8	7	10	15	35	30
abnormal	3	6	7	7	9	15	25	17
normal	0	0	1	0	1	0	10	13
Total number young	14	28	47	24	79	105	272	251

abnormal, while about one-third of the survivors of the gestation period were normal.

Eighty females received in the same shipments as the experimental animals were used as controls. They were fed an adequate stock diet throughout their growing and breeding periods and mated to the same males as the females on the deficient diets. They delivered a total of 104 litters and 822 young. The eyes of 60 of the young, each representing a different litter, were serially sectioned. No retrolental membrane was found in any of the eyes examined. Sixty control offspring were dissected and neither diaphragmatic hernias nor horseshoe kidneys were found. The kidneys of 33 control offspring were found to be normal in serial sections.

DISCUSSION

The experiments reported here represent an attempt to produce congenital malformations in the offspring of rats by maternal vitamin A deficiency. We intended to breed the females in a borderline state of vitamin A deficiency which would alter the development of the offspring without causing intrauterine death of the embryos. As this aim could be realized only in part, because sterility and intrauterine death on one side and normal development of the young on the other side interfered with the experiment, we attempted to regulate some of the factors which improve the yield of abnormal offspring. In our previous communication ('46) only 36 litters were obtained from 140 females. This corresponds to a fertility rate of 25.7%. In the present series the fertility rate was higher, since 114 of 260 females or 43.8% had litters. The increase in fertility rate resulted, however, in the birth of some normal offspring. It was shown in the experiments reported here that the character of the preparatory diet influenced the results to a certain extent. In general, one can expect that improvement of the dietary conditions will result in an increased fertility rate and in a lower percentage of abnormal offspring. While abnormalities of the same type can be obtained in the offspring of mothers who receive any

of the preparatory diets employed, it seems advisable to supplement diet U with 12–25 μg carotene every tenth day.

SUMMARY

Congenital abnormalities can be induced in the offspring of female rats reared and bred on diets deficient in vitamin A. The abnormalities are found in soft tissues and are different from those induced in the skeleton by maternal riboflavin deficiency.

Variations of the preparatory diet influence the yield of abnormal young. Increase of the carotene supplement results in an increased fertility rate, but the percentage of abnormal young decreases. Reduction of the carotene supplements reduces fertility while the percentage of abnormal young increases.

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THE NUTRITION OF THE MOUSE

II. EFFECT OF DIET ON THE BACTERIAL FLORA OF THE INTESTINE AND THE CECUM [1]

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(Received for publication August 26, 1947)

It is well-known that diet influences the intestinal flora of several species of animals, as is shown by the work of Tissier ('08) and later Sanborn ('31) with humans, Belonovsky ('07) with mice, Herter and Kendall ('09–'10) with cats and monkeys, Hull and Rettger ('15, '17) and Porter and Rettger ('40) with rats, and Crecelius and Rettger ('43) with guinea pigs. In general, all of the species responded similarly whenever either of 2 types of diets was fed. Meat or a high protein diet produced proteolytic flora consisting largely of coliform organisms; a variation in the carbohydrate induced a change toward a fermentative flora. For example, milk or lactose, and sometimes dextrin, encouraged an aciduric bacterial population; when dextrin was fed, there was produced a type of flora which synthesized some of the B vitamins (Guerrant et al., '35; Schweigert et al., '45).

Belonovsky ('07) studied only the effect of lactose and milk on the bacteria of the feces and upon the ease of implantation of certain strains of bacteria in the intestine. Little attention was paid to the effect of varying the diet on the predominating

[1] Supported in part by the Nutrition Research Fund of this laboratory and in part by a grant from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. The early experiments in this study were supported by grants from the Nutrition Foundation, Inc., and the Anna Fuller Fund.

flora or the effect that such a change would have in the nutrition of the mouse.

It has been shown in this laboratory that considerable difference exists in the nutritional requirements of several highly inbred strains of mice. In general, the requirements of the C_{57} strain (black, with low incidence of spontaneous mammary tumors) are lower than those for the A strain (albino, highly susceptible to spontaneous mammary tumors). Both of these strains have been used in these studies of the intestinal flora, but this paper deals principally with the A strain.

The studies to be reported here were undertaken to determine the effect of 2 synthetic rations and a natural diet upon the predominating bacteria in the intestinal tract of A strain mice, and whether any such change could affect the nutrition of the host. The lower parts of the small intestine and the cecum were chosen for investigation since it was felt that these were probably the most likely sites of absorption by the mouse of any synthetic products liberated by the bacteria.

The predominating bacteria of these segments of the intestine were studied by Gram stains and by culturing serial dilutions of the contents. Direct bacterial counts were made on the samples and the entire cecal contents were weighed.

EXPERIMENTAL

Weanling mice of the A strain, 21 days old, were placed in individual screen-bottom cages, and food and water supplied *ad libitum*. Growth rates were measured by weighing the animals periodically. The stock ration [2] and 2 synthetic diets (nos. 101 and 133) were used. The composition of the synthetic rations has been described in detail elsewhere (Fenton and Cowgill, '47). Diet 101 contained 23% casein and 60% dextrose, while diet 133 contained 30% casein and 45% dextrin. In other respects the 2 diets were similar except that no. 133 also had cystine, vitamin K, biotin and folic acid added.

Representative animals from these 3 groups were killed by etherization and were opened immediately using sterile tech-

[2] Purina Laboratory Chow.

nique. The cecum and the lower half of the small intestine were removed and the contents from these segments squeezed directly into sterile, tared vials which were then plugged with cotton. The upper part of the small intestine was not studied because of the small numbers of organisms present. The large intestine was not considered to be a likely area of absorption of bacterial excretory products because on the synthetic diets this material formed into hard pellets immediately upon leaving the cecum. It seemed likely that any substances that might be produced by bacteria in this region would be lost to the host. The vials containing the samples were weighed and the samples made up to a known dilution with sterile distilled water. The vials containing the diluted samples (usually a 1 : 25 to 1 : 100 dilution, depending on the size of the sample) were stoppered and shaken thoroughly. Any clumps of contents were broken up with a sterile pipette. The samples were then further diluted to 10^{-4}, and then serially by steps of 100 until a dilution of 10^{-10} was reached.

The cultural work was done first, and the whole procedure after the removal of the contents from the body was done as quickly as possible in order to keep the sample from being exposed unduly to air and low temperatures. The cecal contents were cultured first, as the bacteria isolated from this segment seemed to be more anaerobic than those from the small intestine. Attempts to reduce dissolved air to a minimum consisted of plugging the water blanks with rubber stoppers immediately upon removal from the autoclave, and of boiling the broth for 10 minutes in a water bath, followed by immediate cooling in water just before inoculation. The inoculum was placed carefully under the surface of the broth with a pipette, and the tube was sealed with vaspar.[3] The broth was composed of 1% of each of the following: Bactopeptone, tryptone, peptonized milk, yeast extract, beef extract, and glucose, and 0.5% of K_2HPO_4 all dissolved in liver extract made by extracting 75 gm of Bacto liver powder in a

[3] Composed of equal weights of petrolatum and paraffin.

liter of distilled water. The pH was adjusted to 6.8 before autoclaving.

For the cecal cultures, inocula representing 10^{-8}, 10^{-9}, and 10^{-10} were put into the broth in duplicate while for the small intestine, only dilutions 10^{-8} and 10^{-9} were cultured. The cultures were incubated for 1 week at 37°C. Only these higher dilutions were cultured, as this study was primarily concerned with the predominating organisms. However, in order to learn more about the distribution of coliform organisms, in most cases the E C medium of Hajna and Perry ('43) was inoculated with dilutions of the samples from 10^{-2} to 10^{-7}.

As soon as growth appeared in any tube, the culture was Gram stained, the motility was determined, the pH of the broth was measured electrometrically, and agar shake cultures were made to indicate the degree of anaerobiosis and to permit purification if necessary. The agar shakes were saved so that certain synthetic activities of these cultures could be studied. The results of this phase of the work will be presented in the following paper (Gall et al., '48).

Immediately after the completion of the cultural procedure, a Gram stain was made of the diluted material from the original vial. From this the predominating morphological types and their approximate relation to each other were noted. Direct counts were also made on the sample after suitable dilution. In general the cecal content was diluted 1 : 1000, while the small intestinal content was diluted 1 : 25 to 1 : 100, depending on the expected bacterial count. The slide counts were made on 2 × 2 cm areas by mixing 0.01 ml of the diluted sample with 1 drop of saturated nigrosine solution in methyl alcohol spread evenly over the area. The slides were dried very quickly on a hot plate, and the counts were made with a microscope for which the field had been calibrated. It was easy to see the unstained bacteria against the black background, and from the average of 10 fields on each of 2 slides (about 300–400 organisms), it was possible to calculate the bacterial count per gram of original sample.

The weight of the cecal and small intestinal contents was determined to the nearest milligram on an analytical balance. The material came out of the cecum very easily, and it is felt that the weights obtained represented with fair accuracy the entire cecal contents. The weights of the contents of the small intestines were extremely variable, since the position of the lumps of food in the intestine was so irregular that the inclusion or exclusion of 1 mass of food would alter the result severalfold. Therefore not much significance is attached to these figures, and they are not reported in the table.

In addition to these main experiments on A strain mice a few preliminary studies were carried out on small groups of C_{57} and A strain mice on various other diets under investigation in the laboratory.

RESULTS

Growth rates of male mice fed the stock ration or diet 101 have been reported by Fenton et al. ('47). All 3 diets studied here supported growth about equally well. Fenton and Cowgill ('47) have reported complete failure in securing reproduction and lactation in mice raised and maintained on ration 101. The more complete diet 133 did support reproduction and lactation, but the results were still not as satisfactory as with the stock ration.

Gram stains of the cecal contents of the animals fed these 3 diets were examined microscopically, and the differences in the flora on these diets were sufficiently marked to permit identification of the diet fed, simply by studying the slide. The cecal contents of the animals on stock ration were characterized by the presence of a slender, gram-negative, curved rod and a cigar-shaped organism. Except for some gram-positive rods, probably lactobacilli, there were few other bacteria present. In contrast to this simple flora, the animals on diet 101 harbored a varied bacterial population, but there was always present in these animals a considerable number of large round cocci, occurring in pairs and short chains. This coccus was characteristic of this diet. Diet 133 also supported

a variety of organisms, but the flora differed from that on ration 101 in several ways. The large coccus found on diet 101 was absent, but in its place was found in smaller numbers an elongated coccus, occurring in pairs, and a tiny coccus forming a characteristic, closely united chain of 10–20 organisms. On diet 133 there also appeared in large numbers, a highly curved rod that formed a perfect C.

The Gram stains of the small intestinal contents from animals fed all 3 diets showed the gram-positive rod as the predominating bacterium, but on the 2 synthetic diets the coccus characteristic of that ration usually appeared.

A more complete description of the types of bacteria found on these various diets, as well as some notes on their synthetic activities, especially in regard to B vitamins, will be presented in the following publication.

As shown in table 1 the cultural differences among the 3 groups were also marked and largely confirmed the conclusions drawn from the examination of the slides. The cecal samples cultured from stock diet animals gave scanty growth, due to the predominance of the slender curved rod, an anaerobe which proved very difficult to culture. In no case did the cultures from the animals on this diet grow in as high a dilution as was expected from the slide count. Virtually the only organism isolated from these animals was the easily cultivatable gram-positive rod.

In contrast, the bacteria from the ceca of animals fed diet 101 grew very readily. In 9 out of 12 cases the cultures grew in the dilutions indicated by the slide counts. The characteristic coccus was easily isolated, as were the gram-positive rods, and the coliform organisms when the latter were present. Gas, usually found in 1 or more tubes in the series, could in some instances be attributed to coliforms. However, in some tubes only a slight amount of gas appeared. On staining these cultures only cocci were observed, and attempts to isolate coliforms from these tubes repeatedly failed. This cultural picture identified the animals which had been fed diet 101.

TABLE 1

Summary of the bacterial counts, weights of cecal contents and cultural results obtained from A strain mice.

TABLE 1

Summary of the bacterial counts, weights of cecal contents and cultural results obtained from A strain mice.

DIET NUMBER	501		133		STOCK	
Part of intestinal tract	Small	Cecum	Small	Cecum	Small	Cecum
No. of animals	12	12	12	12	9	9
No. of animals showing agreement of slide and cultural "counts"	12	9	10	3	9	0
Wt. of segment contents, *gm*	..[1]	0.065 ± 0.021[2]	..[1]	0.094 ± 0.029[2]	..[1]	0.407 ± 0.033[2]
Bacterial count — billions						
per gm of sample	0.710 ± 0.81[2]	22.2 ± 11.5[2]	0.324 ± 0.26[2]	23.0 ± 11.8[2]	0.875 ± 0.99[2]	42.8 ± 11.5[2]
per intestinal segment	..[1]	1.5 ± 0.84[2]	..[1]	2.09 ± 1.15[2]	..[1]	17.9 ± 5.4[2]
No. of cultures isolated; according to type						
Gram + rods						
10^{-8}	14	7	9	10	13	13
10^{-9}	7	6	3	5	8	8
10^{-10}	..[1]	3	..[1]	0	..[1]	0
Round coccus						
10^{-8}	7	15	0	0	0	1
10^{-9}	3	9	0	0	0	0
10^{-10}	..[1]	5	..[1]	0	..[1]	0
Elongated coccus						
10^{-8}	0	0	1	6	0	0
10^{-9}	0	0	0	3	0	0
10^{-10}	..[1]	0	..[1]	0	..[1]	0
Tiny coccus						
10^{-8}	0	0	1	3	0	0
10^{-9}	0	1	0	4	0	0
10^{-10}	..[1]	1	..[1]	1	..[1]	0
Curved rod						
10^{-8}	1	0	1	1	0	1
10^{-9}	0	2	0	6	0	1
10^{-10}	..[1]	3	..[1]	1	..[1]	0
Coliforms						
10^{-8}	0	4	0	3	0	0
10^{-9}	0	3	0	0	0	1
10^{-10}	..[1]	3	..[1]	0	..[1]	0
No. of animals showing coliform organisms 100 M or above	..[1]	5	..[1]	2	..[1]	1

[1] Not computed. [2] Standard deviation.

The cecal contents from animals fed ration 133 gave cultural results somewhat different from the other 2 diets. In only 3 cases out of 12 did the cultures grow in the dilution expected from the slide counts, but the discrepancy was not as great as with the stock ration. The elongated coccus and the tiny coccus, which proved to be an anaerobe, occurred in a fair number of cultures. The gram-positive rods were also isolated regularly. The curved, C-shaped rod seen on the smears was never isolated in that morphological form.

It has commonly been thought that coliform bacteria are always among the predominating organisms in the intestinal tract, but this study indicates that with A strain mice its occurrence in large numbers varies with the diet. With the average bacterial count in the neighborhood of 22 billion per gram of cecal contents, coliforms were isolated in dilutions of 1 billion or more only 7 times. Three of these came from the 10 billion dilution and 4 from the 1 billion. These cultures represented 5 animals, 4 of which were fed diet 101. Therefore, in only 10% of the animals tested were coliforms present in the top 2 dilutions, and in only 20% of the cases in the top 3 dilutions. It is pertinent to point out that 41% of the animals on diet 101 had coliforms present in 100 million or more, as compared to much fewer in animals on the other 2 diets. Two-thirds of the animals tested in low dilutions showed less than 1 million coliform organisms per gram of cecal contents and at least 25% were below 10 thousand per gram. It is of interest to note that with the stock ration animals where the opportunity for coprophagy was the greatest, there was the mildest coliform flora.

The cultural results of the small intestinal contents on the 3 diets reflected the findings with the Gram stain. All 3 diets gave a large number of cultures of gram-positive rods, while the 2 synthetic diets showed in addition cocci of the type characteristic of the ration.

The 3 diets were compared as to bacterial count per gram of cecal material and total bacterial count per cecum. For reasons stated above, a similar study for the small intestinal

contents is not reported in table 1. The animals eating the stock ration showed a higher bacterial content when measured per gram or per cecum, although the difference is greater by far when measured by the latter criterion. This is a function of the greater content of the cecum of the animals eating the natural diet. By statistical analysis these differences were found to be highly significant ($p < 0.01$). There was little difference between the animals eating the 2 synthetic diets, however. Statistically there was no difference between the cecal count per gram on diets 101 and 133. A comparison of the total cecal counts of these diets also showed no significant difference ($p = 0.2$).

The cecal contents obtained from animals fed the stock ration were statistically highly significantly heavier than the contents from the animals on the 2 synthetic diets ($p < 0.01$). The animals on diet 133 showed greater cecal contents than those on 101 (p just equals 0.01). This should be interpreted cautiously, however, for reasons to be discussed below.

By way of preliminary investigation a few animals of both C_{57} and A strains were studied after having been maintained on the following diets: (a) 3 diets similar to no. 133, but with dextrose as the carbohydrate, and a diet identical with no. 101 (containing dextrose), but omitting roughage; (b) a diet low in riboflavin and one low in pantothenate, but in other respects similar to no. 101; (c) a diet similar to no. 101 with 1% sulfathalidine added; (d) a ration identical with no. 101, but replacing the dextrose with dextrin.

The diets listed under (a) showed a cecal flora essentially the same as that found on ration 101. The animals deficient in riboflavin and pantothenate showed the same cecal flora as those on diet 101 except that a C-shaped rod was also present. The mice fed sulfathalidine showed a flora similar to those on ration 101, but the weights of their cecal contents were somewhat greater. When the dextrose of diet 101 was replaced with dextrin, the resulting flora resembled that which was found on the dextrin containing diet no. 133. In all of these preliminary studies it must be emphasized that only a micro-

scopic examination was made, thus affording a comparison on the basis of morphology alone.

A group of mice was fasted 24 hours and then fed the stock ration. Small numbers of these animals were sacrificed and their cecal contents studied at various intervals following feeding. The animals sacrificed 3.5 to 6 hours after feeding showed a C-shaped rod, which was not present in animals permitted to feed until sacrificed or in mice sacrificed up to 2.5 hours after feeding, following a period of fasting.

DISCUSSION

Ample evidence has been presented in the main portion of this study and also in the preliminary work reported here showing that the composition of the diet can have a marked effect upon the intestinal flora of the experimental animals. The bacteria seen on each of the 3 rations investigated have been so distinctive that it was possible to judge by microscopic examination alone which diet had been fed to any particular animal. In fact, following the initial experiments, we made it a practice to give the investigator (L.S.G.) an animal for the day's work without her having any knowledge of the nature of the ration. On the basis of the Gram stain, the examiner would attempt to identify the diet fed, and in only 1 case out of 33 was an error made.

Many investigators have implied that a great deal of the intestinal synthesis should be ascribed to coliform bacteria. Our data do not warrant such conclusions, at least for this highly inbred strain of mouse. We have found appreciable numbers of coliforms only on diet 101, and here only 5 out of 12 animals showed this organism in the top dilutions.

It is important to stress that with the animals fed the stock ration and diet 133, we frequently failed to obtain cultural results commensurate with the slide count. More work is necessary to determine the cultural requirements of the bacteria which predominate on this diet.

In view of the greater roughage content of the stock ration, it was to be expected that animals on this diet would have

greater cecal contents than the animals on synthetic diets. In our studies comparing 2 synthetic diets, 1 containing dextrin (no. 133) and the other dextrose (no. 101), there was a statistically significant difference between the weights of the cecal contents of the 2 groups (p just equals 0.01). However it must be stated that the animals fed diet 133 were slightly older than those eating no. 101, and for this reason the results should be interpreted with caution. Mannering et al. ('44) found that rats fed a synthetic diet containing dextrin had a larger cecal content per unit of body weight than animals fed a similar diet containing sucrose.

It must also be stated in this connection that 6 of the 9 mice fed the stock ration were older than the animals on synthetic ration, while 3 were the same age or younger. On comparison the average weights of the cecal contents of the young and old animals on stock ration were found to be so nearly alike ($0.386 \pm .038$ and $0.417 \pm .045$, respectively), that it was felt that these age differences did not seriously affect the interpretation.

The study reported here on both C_{57} and A strains of mice on different diets gives evidence that there is a flora, characteristic of mice fed a dextrose diet, which is different from that of animals maintained on a ration containing dextrin. This is in agreement with the findings of other workers on several species cited in the introduction, that certain carbohydrates induce differences in intestinal flora.

SUMMARY AND CONCLUSIONS

1. Microscopic and cultural examinations of the small intestine and the cecum of A strain mice on stock ration and 2 synthetic diets were carried out.

2. Mice fed each of these diets had a characteristic cecal flora; on the stock ration it consisted of a slender, gram-negative, curved rod; on the 2 synthetic diets cocci characterized each of them.

3. Coliforms were not found to be present in significant numbers on 2 of the diets and were found in large numbers in less than half of the mice on diet 101.

4. The mice on the stock diet had a larger bacterial population per gram of cecal contents and per cecum than the mice on synthetic diets. There was no significant difference between the 2 groups of mice on synthetic diets.

5. The weights of the cecal contents of the animals on stock ration were greater than those of the animals fed synthetic diets. There was little difference between the animals on synthetic diets with respect to weights of cecal contents.

ACKNOWLEDGMENTS

We wish to acknowledge gifts of B-complex vitamins from Merck and Co., Inc., Hoffmann-LaRoche, Inc., and Lederle Laboratories, Inc. The technical assistance rendered by Marie A. Stone was greatly appreciated.

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THE NUTRITION OF THE MOUSE

III. RELATION OF DIET TO THE SYNTHETIC ACTIVITY OF THE PREDOMINATING FLORA ISOLATED FROM THE SMALL INTESTINE AND CECUM [1]

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(Received for publication August 26, 1947)

It has been established that the bacteria of the intestinal tract are active in the synthesis of certain B vitamins (Mitchell and Isbell, '42), and it has been demonstrated that the cecum is probably the site of this synthesis (Taylor et al., '42; Schweigert et al., '45). It has also been shown that certain carbohydrates such as dextrin promote greater intestinal synthesis of these B vitamins than the simpler sugars, sucrose and dextrose (Guerrant et al., '35; Schweigert et al., '45).

In most of these studies, the synthetic activities of the whole intestinal flora as found *in vivo* have been investigated, with little attempt to isolate and study the synthetic activities of the predominating flora. Also since it is the vitamin content of the fecal flora that has been the subject of several studies, it has not been clearly demonstrated whether the vitamins are available to the host, unless coprophagy is practiced or considerable autolysis occurs in the intestinal tract.

[1] Supported in part by the Nutrition Research Fund of this laboratory, and in part by a grant from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. The early experiments in this study were supported by grants from the Nutrition Foundation, Inc., and the Anna Fuller Fund.

The purpose of our preliminary studies has been to determine whether any of the predominating organisms isolated from the small intestines and ceca of mice fed different diets showed any marked differences in their synthetic activities with respect to the B vitamins, especially the occurrence of the vitamins in the medium surrounding the bacterial cells. We have observed in this laboratory (Fenton and Cowgill, '47a, b) that mice of the C_{57} and A strains differed in their requirements for riboflavin and pantothenic acid. It seemed to us that possibly this difference could be attributed to differences in the rates of intestinal synthesis.

EXPERIMENTAL

The methods for maintenance of the mice and for isolation of the predominating flora have been presented in the preceding paper (Gall et al., '48). A description of the diets or a reference to their composition will also be found in that paper.

The cultures were obtained from 62 mice of the A and C_{57} strains fed 7 different diets, which can be grouped into 3 main classes: a stock diet and 2 synthetic diets, 1 containing dextrin and the other dextrose. This study included the testing of a total of 107 cultures, of which 83 came from the 3 top dilutions of the cecal contents, 18 from the 2 highest dilutions of the small intestinal contents, and 6 were coliform organisms from lower dilutions of contents from both intestinal segments.

The organisms were tested within 1 week of isolation in most cases. Cultures for testing were obtained by planting a single colony from an agar shake culture prepared from the original broth into a complete synthetic broth (Krehl and Illingworth), modified by using the casein hydrolysate described by Roberts and Snell ('46). Since most of the original cultures showed only 1 type of bacterium in the Gram stain, little difficulty was experienced in obtaining a pure culture. In those few cases where a tested organism came from a mixed culture, a series of agar shake cultures was made to insure a well-isolated colony. To check for purity, Gram stains

were made from the broth planted with the single colony, and from the culture representing the third transfer in complete broth.

After the culture in complete broth had been incubated for 24 hours at 37°C., it was observed for growth. If turbidity was apparent, 0.1 ml of the culture was used as an inoculum for the various broths employed to test its synthetic abilities. The media inoculated were as follows: a complete synthetic broth of the same composition as that in which the organism had been growing, and 5 deficient broths, identical with the complete broth, except for the omission of 1 of the following vitamins from each broth — riboflavin, niacin, biotin, folic acid and pantothenic acid.

All broths for the series of 3 transfers and the subsequent microbiological assays on these cultures were prepared simultaneously once each week. Three serial transfers were made of each culture at 24-hour intervals, each culture being transferred into a broth identical with the one on which it was growing. Immediately prior to the inoculation with 0.1 ml of the culture, the broths were autoclaved and cooled rapidly. All anaerobic cultures were sealed with vaspar. The growth of the cultures was graded from 1 + to 4 +, indicating the degree of turbidity produced. If a tube failed to show visible growth before the third transfer, it was carried for one more transfer before being discarded.

A slightly different procedure was used for the coliforms after it became apparent that the synthetic broth referred to above would not support satisfactory growth of the coliforms. A mineral salt and glucose broth (MacLeod, '40) was used for the serial transfers.

If the culture survived 3 transfers in any of the deficient broths, the supernatant fluid obtained by centrifuging the 24-hour culture was tested for the presence of the omitted vitamin. This was done by microbiological assay, following the procedure of Snell ('47) except for a modification of the broth. The synthetic medium used in this study was the broth of Krehl and Illingworth (unpublished data), modified as

noted above. The assay was carried out on 1, 2 and 4 ml of the supernatant broth, using *L. casei* as the test organism for riboflavin and folic acid, and *L. arabinosis* for niacin, biotin and pantothenic acid. No regular standard curve was run — only a blank and a maximum tube — as it was not desired to estimate the amount of B vitamin quantitatively, but merely to determine whether large or small amounts of the vitamin were liberated from the cells. The results were graded on a scale running from 1 + to 4 +. The 1 + and 2 + values were interpreted as indicating slight synthesis and liberation of the vitamin, while 3 + and 4 + values meant that the titration approached or equalled that of the maximum tube, indicating considerable amounts of the vitamin to be present in the broth. A value was not considered significant unless the titration of the tube containing 1 ml of the supernatant fluid was greater than the blank, and unless the titration increased as the amount of the added supernatant fluid increased.

RESULTS

In general the predominating organisms isolated from the intestinal contents of the mice on the different diets were of 7 types. A table showing the distribution of 6 of these types isolated from A strain animals on different diets will be found in the preceding paper (Gall et al., '48). The seventh type occurred mostly in 10 out of 12 black mice on diet 101, and will be discussed here. The distribution of the other organisms from the C_{57} mice was about the same as that for the white mice on the same diets.

A summary of a few characteristics of the bacteria isolated is presented in table 1. None of the organisms isolated was motile or formed spores, so these characteristics were not included in the table. No attempt has been made to key out these bacteria, but some tentative genus names will be suggested for 3 of the types of organisms. The gram-positive rods were probably Lactobacilli, and the gram-negative anaerobic rods, both the curved and the short fat ones, resembled certain of the Bacteroides as described by Eggerth and Gag-

TABLE 1

Some characteristics of 7 types of bacteria isolated from high dilutions of small intestinal and cecal contents of mice on several different diets.

ORGANISM TYPE AS DESIGNATED IN TEXT	MORPHOLOGY	GRAM REACTION	OXYGEN RELATION	GROWTH IN ORGANIC BROTH	FINAL PH IN ORGANIC BROTH	GAS PRODUCTION	GROWTH IN SYNTHETIC BROTH	DISTRIBUTION ON VARIOUS DIETS
Gram-positive rod	single or short chains	positive	facultative	moderate 16 hrs.	5.5–6.0	negative	poor	all diets
Curved rod	single or pairs	negative	anaerobic	slight 3–4 days	6.3–6.4	negative	poor	all diets
Short, fat rods	single	negative	anaerobic	moderate 2–3 days	6.0–6.3	negative	absent	mostly black mice on diet 101
Coliforms	single	negative	facultative	heavy, 16 hrs.	5.3–5.4	heavy	poor	mostly white mice on diet 101
Tiny coccus	long, closely united chains	positive	anaerobic	slight 2–3 days	5.9–6.0	negative	absent	mostly diet 133; few on 101
Round coccus	short chains and masses, pairs	positive	facultative	heavy, 16 hrs.	4.5–4.7	slight?	good	dextrose containing diets
Elongate coccus	pairs and long chains	positive	facultative	heavy, 16 hrs.	4.9–5.3	negative	good	dextrin containing diets

non ('33). The gram-positive organisms usually exhibited that reaction only in young cultures, and the cocci frequently appeared gram-negative in the original stain from the intestinal contents. The overgrowth of the slower growing anaerobes by the faster growing bacteria explains the occurrence of these anaerobes in the top dilution only, where they are found after the competing organisms have been diluted out.

These 7 types of bacteria were tested for their ability to survive 3 transfers in the absence of certain of the B vitamins omitted singly from an otherwise supposedly complete broth, and if they survived, to contribute to this broth measurable amounts of the vitamin omitted, as shown in table 2.

It will be observed from this table that 2 of the types of organisms never grew in the synthetic broth, and therefore their synthetic activities could not be tested. Two more of the bacterial types grew poorly when they survived at all. The curved rod was subcultured only twice, but both times it survived the 3 transfers in the pantothenic acid deficient broth, and was shown to liberate this vitamin into the broth, in 1 case in large quantities. The gram-positive rods that grew readily in the organic medium proved difficult to culture in the synthetic broth, and although one-third of the trials to obtain 3 transfers in synthetic broth were successful, the growth was weak. In a few cases small amounts of one of the B vitamins were found to be present in the broth. The poor growth might account for the slight synthetic activity observed.

Oddly enough the coliforms grew poorly in this synthetic broth. Known strains of E. coli [2] as well as the recently isolated cultures, gave correspondingly poor results. When the simpler, mineral salt broth was employed, growth was better, and the supernatant broth of these cultures was found to contain considerable quantities of all 5 vitamins.

A striking difference appeared when we compared the activity of the 2 facultative cocci. They both grew well in the synthetic broths, both complete and deficient, with the ex-

[2] Obtained through the kindness of Dr. P. B. Cowles, Department of Bacteriology, Yale University.

TABLE 2

Summary of the ability of 7 groups of intestinal bacteria to synthesize and liberate 5 B vitamins.

TYPE OF ORGANISM	NO. OF CULTURES TESTED	NO. SURVIVING 3 TRANSFERS IN COMPLETE BROTH	INTENSITY OF GROWTH IN BROTH	NO. SURVIVING 3 TRANSFERS IN BROTH DEFICIENT IN						DEGREE OF LIBERATION OF VITAMIN	NO. OF TIMES VITAMIN FOUND IN SUPERNATANT BROTH				
				Complete	B_2	Niacin	Biotin	Folic acid	Pantothenic acid		B_2	Niacin	Biotin	Folic acid	Pantothenic acid
Gram positive rod	32	11	+	11	11	1	11	7	8	+	1	0	2	2	0
Curved rod	12	2	+	2	1	1	0	0	2	+	0	0	0	0	1
										+++	0	0	0	0	1
Short fat rod	4	0													
Tiny coccus	7	0													
Round coccus	30	28	+ to ++	15	18	21	16	6	14	+ to ++	7	2	2	3	1
			+++ to ++++	13	7	0	4	3	4	++++	0	0	0	1	0
Elongate coccus	8	8	+ to ++	3	6	8	5	3	6	+ to ++	4	1	2[1]	0[1]	0
			+++ to ++++	5	2	0	3	5	1	+++ to ++++	0	0	0	6	0
Coliforms	14														
regular synthetic broth	4	3	+ to ++	3	2	3	2	3	2	+ to ++	1	3	0	0	0
										+++ to ++++	1	0	0	2	0
mineral salt broth	10	9	+++	9	(mineral broth only) no vitamins added					+ to ++	4	5	3[1]	0	2
										+++ to ++++	4	1	3	9	5

[1] One culture in group spoiled while testing.

ception of the medium without folic acid. In this broth the coccus from animals on diets containing dextrin grew as well as on the other synthetic broths, while only 9 out of 28 cultures of the coccus from the mice fed dextrose-containing diets survived the 3 transfers in this medium, and 6 of these showed only weak growth. This difference is further emphasized by noting the data on the presence of folic acid in the supernatant broth after the cocci had been planted in the folic acid deficient broth. Of the 7 cultures tested from animals fed diets containing dextrin, 6 showed the presence of large amounts of folic acid in the supernatant broth. In contrast, the cocci from mice fed dextrose-containing diets, showed liberation of folic acid in only 4 cultures, and of these, only 1 showed the presence of large amounts.

DISCUSSION

The results of this preliminary study tend to indicate that differences in diet influenced the bacterial synthesis of B vitamins by the alteration of the organisms present in the intestinal tract. However, this is far from the final answer, as will be pointed out in the following discussion.

There are 2 major weaknesses in this work. The first, which is largely unavoidable with our present culturing techniques, is the valid criticism that the *in vitro* findings do not necessarily reflect the *in vivo* activities of the organism. The second weakness lies in the inability to isolate some of the predominating organisms at all, or to subculture a bacterium in synthetic broth even after primary isolation, thereby making the study of their synthetic activities impossible. Obviously further work is needed on this aspect of the problem.

This indicates that in this study, the techniques employed failed to meet the cultural requirements of all of the predominating flora. This proved to be especially true of the cecal bacteria. The conditions were more nearly met with the rich organic broth than with the synthetic medium, however, as was shown by the inability to subculture some of the primary isolations in synthetic broth, although they could be success-

fully transplanted into organic media. This suggests that for these bacteria, the synthetic broth may have had a deficiency or an imbalance.

However, the poor growth or lack of growth of some organisms in the rich organic medium indicates that there are still other flaws in the present technique. This may well be remedied by improving the means of maintaining anaerobiosis, as many of the cecal organisms were shown to be obligate anaerobes. Further work should be done in the field of culturing these organisms.

Several considerations, to be discussed here, entered into the development of the technique as it was used in these studies. In the preceding paper we have discussed our reasons for choosing the lower small intestine and the cecum for our study of their flora, since it would seem that in these areas any vitamins liberated from the cells would have the best chance of being absorbed by their host. It was also because of considerations of availability to the host that we selected the supernatant fluid of the cultures to test for the presence of the vitamins. Any vitamins held in the cells would be available to the host only if he practiced coprophagy, or if active autolysis took place in the intestinal tract at or above a point where absorption could take place. As the degree of cellular breakdown which takes place in such a region is uncertain, we tried to minimize autolysis as much as possible by using young cultures (24-hour) for testing. In most cases our cultures were increasing in turbidity between the eighteenth and twenty-fourth hour, suggesting an actively growing culture rather than a static one in which autolysis would be prevalent.

In these studies we have considered only the predominating flora of the cecal and small intestinal contents, as we felt that the bacteria present in the largest numbers would have the greatest chance to make a significant contribution to their host by any liberation of vitamins.

As has been pointed out in the preceding paper, we have not regularly found coliforms to be present as a predominating

organism except in a few white mice fed diet 101. In most cases these organisms appeared only at a much lower level of dilution. It does not seem likely that the coliforms, in spite of their good synthetic activities with respect to the 5 B vitamins tested (Thompson, '42; Burkholder and McVeigh, '42; also this paper), could contribute much to the nutrition of the mice maintained under the conditions of this study, with the exception of the few mice fed diet 101, mentioned above.

The relationship of the presence of coliforms to diet is only 1 instance of the role that diet plays in influencing the type of intestinal flora of the mouse. The difference in the type of cocci isolated from mice fed dextrose- or dextrin-containing diets is further evidence of the effect of the carbohydrate in the ration on the intestinal bacteria isolated. The importance of this difference in cocci is brought out further by the striking difference in their synthetic activities with respect to folic acid. From these results, we can assume that the coccus isolated from animals on a dextrin-containing diet was probably supplying folic acid to its host. In contrast, the coccus isolated from mice maintained on a diet containing dextrose was not elaborating any significant amount of folic acid, but apparently actually required it for growth. It was therefore probably competing with its host for any folic acid present in the diet or liberated from any other intestinal organism. Such competition for this essential nutrient might even prove detrimental to the host.

Riboflavin was found to be present in small amounts in the supernatant fluid of about one-fifth of the cultures tested. Since it has been shown (Mitchell and Isbell, '42) that riboflavin is not readily liberated from the cell, this is in keeping with the work of other investigators in this field. Although the amount liberated by each organism was small, it may be enough to become of importance when an animal is on a marginal riboflavin intake. Also any factor which would increase the number of these organisms, such as an increase in bacterial population in the cecum, should correspondingly increase the total amount of riboflavin produced. The difference in the ribo-

flavin requirement between the C_{57} and A strain mice (Fenton and Cowgill, '47a) might be explained by the difference in bacterial population in the cecum (Gall et al., '47). A similar explanation might apply to the differences in requirements of these 2 strains for pantothenic acid (Fenton and Cowgill, '47b), since the curved rod which is a predominating organism on all 3 diets, liberated pantothenic acid both times it was successfully cultured in synthetic broth.

On the basis of these preliminary studies, it might be said that diet may influence the nutrition of the host indirectly by affecting the intestinal flora, as well as by supplying the nutrients directly.

SUMMARY AND CONCLUSIONS

1. A study was undertaken to determine the ability of 107 cultures, isolated from high dilutions of intestinal contents of mice fed 7 different diets, to synthesize and liberate from their cells 5 vitamins — riboflavin, niacin, biotin, folic acid and pantothenic acid, and to determine whether the synthetic products of the flora were influenced by diet.

2. A partial description was given of 7 different types of bacteria found in the top dilutions of intestinal contents of mice, with a notation as to their distribution on the various diets.

3. Differences were observed in the ability of these cultures to grow in synthetic broths, both complete and deficient in 1 of the 5 vitamins. In some instances these differences were correlated with diet, as in the case of 2 cocci. The coccus characteristic of the flora found in mice fed dextrin-containing diets, grew well in the synthetic broth lacking folic acid, in contrast to the coccus found in animals fed a diet containing dextrose, where little or no growth was observed in the folic acid-deficient broth.

4. These 2 cocci also exhibited differences in the extent of folic acid liberation; the coccus which grew well on the broth lacking folic acid liberated large amounts of this vitamin into the environment, while the coccus which grew poorly, or not

at all, in the folic acid-deficient broth, liberated little if any of this vitamin.

ACKNOWLEDGMENTS

Gifts of B vitamins were received from Merck and Co., Inc., Hoffmann-LaRoche, Inc., and Lederle Laboratories, Inc.

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EFFECTS OF B VITAMINS, LIVER AND YEAST ON GROWTH UNDER COLD ROOM AND ROOM TEMPERATURE CONDITIONS [1]

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(Received for publication September 18, 1947)

Available data indicate that liver contains at least 1 factor other than the known B vitamins essential for optimal growth in the immature rat (Bosshardt, Ayres, Ydes and Barnes, '46; Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47; Cary, Hartman, Dryden and Likely, '46). Weanling rats fed purified rations containing liver invariably gained more weight than animals fed similar diets containing the B vitamins in synthetic form. These differences, although consistent for all experiments cited above, were not sufficiently marked in most cases to be statistically significant (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47), particularly with diets containing unextracted casein (Cary, Hartman, Dryden and Likely, '46). The present experiment was undertaken to determine whether the above differences in growth might not be accentuated by raising animals under conditions of cold. Available data indicate that body requirements for essential nutrients may be increased by physical exertion,

[1] The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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fever, thyroid-feeding and other conditions resulting in an increased metabolic rate (Ershoff, '48). Such "stress factors" raise body requirements beyond the usual or average range, accentuate deficiencies and hasten the onset of symptoms on a deficient diet. It was felt that since excessive doses of thyroid caused retarded growth preventable by liver feeding in the rat (Ershoff, '47a, '47b), exposure to cold with its attendant rise in oxygen consumption might also serve as a "stress factor" resulting in increased requirements for unknown growth factors in the rat.

PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. Ninety female rats of the Long-Evans strain were selected at 23 to 25 days of age at an average weight of 55 gm. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the diets listed in table 1. Feeding was continued for 8 weeks. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C.[3] and (2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. Food consumption was determined daily for the first 4 weeks of feeding. Animals were autopsied after 8 weeks of feeding, organ weights determined, the ovaries and thyroids fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin. A second experiment was conducted under conditions similar to the above except for a cold room temperature of 6 ± 2°C. in contrast to the 2 ± 1.5°C. employed above. Forty-eight

[3] Animals were kept continuously in the dark except for such times as they were being fed or weighed. Previous work indicates that exclusion of light is without significant effect on resulting pathology under cold room conditions (Kenyon, '33).

female rats of the Long-Evans strain were weaned at 21 to 23 days of age and fed diets A, C and D, both under cold room and room temperature conditions. Feeding was continued for 8 weeks. During the eighth week of feeding metabolic rates were determined for all rats in both the cold room and room temperature series.

TABLE 1

Composition of experimental diets.

DIETARY COMPONENT	DIET A	DIET B	DIET C	DIET D
Yeast [1]	0.0	0.0	10.0	0.0
Whole liver powder [2]	0.0	0.0	0.0	10.0
Vitamin test casein [3]	22.0	22.0	22.0	22.0
Salt mixture [4]	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5

To each kg of diet A, C and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2 methyl-naphthoquinone 5 mg and choline chloride 1.2 gm.

To each kg of diet B were added thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, inositol 1.2 gm, p-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-naphthoquinone 10 mg and choline chloride 1.2 gm.

Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A–D concentrate [5] containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

[1] Brewers' Type Yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo.

[2] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

[3] Vitamin Test Casein, General Biochemicals, Inc., Chagriu Falls, Ohio.

[4] Salt Mixture no. 1 (Sure, '41).

[5] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

Findings are summarized in table 2. Data for the cold room series were computed on the basis of the top 8 animals in each group (originally 12 rats per group) in the first experiment and for the top 7 animals per group (initially 10 rats per group) in the second experiment to minimize variations in averages due to early deaths, infection and atypical responses on the part of individual rats. Growth was significantly reduced in all rats under cold room conditions; animals fed

liver (diet D), however, gained significantly more weight than those fed synthetic rations (diets A and B) with growth on the yeast-containing ration (diet C) intermediate between the two.[4] At room temperature findings confirm those of earlier workers (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47). Animals fed liver gained more weight than

TABLE 2

Effects of B vitamins, liver and yeast on growth of rats maintained under cold room and room temperature conditions.

GROUP DIETARY	FIRST EXPERIMENT			SECOND EXPERIMENT		
	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period[1]	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period[1]
	Cold room series					
		gm	*gm*		*gm*	*gm*
A	8	54.4	88.4 ± 2.8	7	50.1	84.9 ± 3.1
B	8	56.0	93.0 ± 3.9			
C	8	55.6	99.3 ± 3.8	7	49.8	91.6 ± 3.7
D	8	56.0	111.5 ± 5.0	7	50.3	112.7 ± 4.8
	Room temperature series					
A	6	53.8	146.0 ± 7.3	6	49.6	142.3 ± 8.1
B	6	53.9	155.4 ± 9.6			
C	6	53.7	159.0 ± 8.1	6	49.4	152.8 ± 8.5
D	6	53.7	167.7 ± 9.9	6	49.8	160.5 ± 10.2

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

those fed synthetic or yeast-containing rations. At room temperature, however, these differences were in no case sufficiently pronounced to be statistically significant.

Data on food consumption and the relative efficiency of the various diets for the building of body tissue are summarized in table 3. The findings indicate that for the first 4 weeks of feeding relative efficiency as measured by the ratio of the gain

[4] The highest values obtained on diet A were less than the average on diet D. In both experiments the quotient of M.D./S.E.M.D. for diets A and D was in excess of 4. Values of 3 or larger are considered statistically significant.

TABLE 3

Summary table showing average gain in weight, average food consumption and ratio of increase in weight to calories consumed for the first 28 days of feeding.

DIETARY GROUP	INITIAL BODY WEIGHT	AVERAGE GAIN IN BODY WT.[1]	FOOD CONSUMPTION (GM/DAY) ON FOLLOWING WEEKS OF EXPERIMENT[2]				AVERAGE TOTAL FOOD INTAKE PER RAT FOR FIRST 4 WEEKS OF FEEDING[1,2,3]		EFFICIENCY[4]
			1st	2nd	3rd	4th			
Cold room series (8 animals per group)									
		gm					*gm*	*calories*	
A	54.4	51.8 ± 2.3	12.7	14.8	15.5	16.9	419.6 ± 8.5	1552.5 ± 31.5	3.31 ± 0.18
B	56.0	54.0 ± 2.3	13.2	16.9	17.1	18.0	457.0 ± 9.2	1690.9 ± 34.0	3.28 ± 0.11
C	55.6	53.8 ± 3.4	12.8	15.5	16.2	17.9	435.2 ± 11.1	1610.2 ± 41.1	3.37 ± 0.17
D	56.0	63.7 ± 3.3	13.0	16.4	17.1	18.0	451.1 ± 12.3	1669.0 ± 45.5	3.88 ± 0.09
Room temperature series (6 animals per group)									
A	53.8	91.3 ± 4.6	8.9	10.5	11.6	11.7	298.8 ± 3.7	1105.6 ± 13.7	8.19 ± 0.41
B	53.9	104.7 ± 5.2	9.5	11.1	12.6	13.2	324.6 ± 12.8	1201.0 ± 47.5	8.70 ± 0.20
C	53.7	111.5 ± 5.0	9.6	11.6	12.5	13.0	325.8 ± 13.6	1205.5 ± 50.4	9.22 ± 0.28
D	53.7	120.2 ± 8.6	9.8	12.2	13.2	13.7	341.8 ± 16.1	1264.7 ± 59.2	9.45 ± 0.30

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] These values are based on basal ration only and do not include the cottonseed oil ingested in supplementary feedings.

[3] The caloric value of diets A, B, C or D approximated 3.7 calories per gram of basal ration.

[4] $\frac{\text{Gm increase in weight}}{\text{Calories consumed}} \times 100$.

in weight × 100 to the calories consumed was slightly higher for animals fed liver (diet D) both under cold room and room temperature conditions than for those fed other diets tested. In most cases, however, these differences were not statistically significant. Values for the cold room series were less than for animals maintained at room temperature conditions. These differences were due, at least in part, to increased heat loss under cold room conditions.

TABLE 4

Body and organ weights of rats maintained under cold room and room temperature conditions.

DIETARY GROUP	INITIAL BODY WEIGHT	BODY WEIGHT AFTER 8TH WEEK OF FEEDING	VENTRICULAR WEIGHT	KIDNEY WEIGHT	OVARIAN WEIGHT	ADRENAL WEIGHT
Cold room series (8 animals per group)						
	gm	*gm*	*mg*	*gm*	*mg*	*mg*
A	54.4	142.8	785	1.678	35.4	40.0
B	56.0	149.0	733	1.634	37.0	36.6
C	55.6	154.9	770	1.832	32.5	39.2
D	56.0	167.5	826	1.868	40.3	51.1
Room temperature series (6 animals per group)						
A	53.8	199.8	640	1.602	46.1	46.0
B	53.9	209.3	659	1.664	52.7	44.2
C	53.7	212.7	637	1.673	55.5	49.7
D	53.7	221.4	649	1.758	54.3	59.5

At autopsy significant differences were observed between animals raised under cold room and room temperature conditions (table 4). Not only was growth retarded in the former series but approximately half the rats in this group lost part or all of their tails during the 8-week feeding period;[5] ventricular weight was increased not only relative to body weight but in absolute weight as well; kidney weights were increased relative to body weight; thyroids were similar to those described by Kenyon ('33) with cellular hypertrophy and loss of colloid; and approximately one-third of the rats

[5] Apparently due to poor circulation in the tail with resulting necrosis and sloughing off of the affected tissue.

had ovaries infantile both in weight and microscopic appearance. With the exception of growth, animals raised under cold room conditions did not differ significantly from one another on any of the diets employed.

Data on oxygen consumption for animals raised under cold room and room temperature conditions are summarized in table 5.[6] The apparatus employed in making these determinations was a closed circuit type with a capacity of 2 liters

TABLE 5

Oxygen consumption of rats maintained under cold room and room temperature conditions.

DIETARY GROUP	NUMBER OF ANIMALS	O_2 CONSUMPTION ML/HR/100 GM BODY WEIGHT[1]	INCREASE OVER B.M.R.[2]
Cold room series			
			%
A	7	252 ± 17	92.4
C	7	205 ± 12	50.7
D	7	198 ± 8	55.9
Room temperature series			
A	6	131 ± 5	...
C	6	136 ± 5	...
D	6	127 ± 4	...

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

Values for O_2 consumption in ml/hr/100 gm body weight are somewhat greater for the room temperature series than those usually reported as basal for the rat. These differences may be due in part to a strain difference.

[2] Compared to the control B.M.R. in the room temperature series.

(Mason and Winzler, '47). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. Readings were made during the eighth week of feeding, and for the cold room series at a temperature of 6°C. with apparatus present in the same room in which animals were housed. In the room temperature series respiration chambers

[6] We are indebted to Mr. G. D. Mason for these determinations.

were kept at 28°C., and in both cases readings obtained were corrected to standard temperature and pressure. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum. Findings in table 5 indicate that average oxygen consumption in ml/hr/100 gm body weight was at least 50% greater for the cold room rats than were values obtained at 28°C. for the room temperature series.[7] In the cold room series values for diet A were greater than for rations containing liver or yeast; there is a question, however, whether these differences are significant. In the room temperature series no significant difference in metabolic rate was noted on the various diets employed.

DISCUSSION

Since the differences in growth between the synthetic and liver-containing diets were significant under cold room but not room temperature conditions, it is felt that the increased metabolism resulting from exposure to low environmental temperature increased requirements for 1 or more nutrients present in liver but not present in significant amounts in other diets employed. The increased requirements under cold room conditions accentuated tissue deficiencies on the synthetic diets employed and resulted in a gain in body weight significantly less than that observed when liver was added. Findings in the present experiment indicate that the protective factor was distinct from any of the known B vitamins and that it was not present in significant amounts in yeast. Liver, however, counteracted only part of the growth retardation observed under cold room conditions. This finding indicates either that (1) insufficient amounts of liver were employed, (2) the retardation of growth was due to factors other than of nutri-

[7] The increase in metabolism due to exposure to low environmental temperature was less in the present series than that reported by other investigators (Benedict and MacLeod, '29). This difference may be due in part to adaptation during our prolonged feeding period as well as to possible differences in the strain of rat employed.

tional origin, or (3) that the animals raised under cold room conditions were deficient in nutrients other than those listed above.

Available data indicate that rats raised under cold room conditions differed in several important respects from those fed excessive doses of thyroid although in both cases oxygen consumption was significantly increased. Both growth retardation and total caloric intake were significantly greater for animals maintained at cold room conditions than for those fed massive doses of thyroid, although oxygen consumption in ml/hr/100 gm body weight was greater for the latter (Ershoff, '47b). In both groups ventricular weight was increased, but no reduction was observed in myocardial creatine under cold room conditions (Brunish and Ershoff, '47) such as occurred in animals fed thyroid (Bodansky, '35; Ershoff, '47b). Finally, liver completely counteracted the retardation of growth of animals fed massive doses of thyroid (Ershoff, '47a, '47b), while such was not the case with the retarded growth occurring under cold room conditions.

SUMMARY

Immature female rats were raised to maturity under cold room and room temperature conditions on purified rations containing the B vitamins as synthetic factors and as present in whole liver and yeast. Growth was markedly reduced in all rats under cold room conditions; animals fed liver, however, gained significantly more weight than those fed other diets employed. The protective factor(s) was distinct from any of the known B vitamins and was not present in significant amounts in yeast. At room temperature conditions no significant difference in growth occurred on any of the diets tested. The suggestion is made that whole liver contains at least 1 factor other than the known B vitamins whose requirement is increased in animals maintained under cold room conditions.

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EFFECT OF VEGETARIAN SELF-SELECTION DIETS ON REPRODUCTION AND THE GROWTH OF OFFSPRING OF RATS[1]

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(Received for publication September 11, 1947)

In a preceding paper ('47), we described vegetarian self-selection diets (diets 6, 6C and 6CC) which were used in studies of the growth and longevity of rats. These diets differed only in regard to the fresh leafy vegetable supplements that were used. Diet 6 was supplemented mainly by lettuce, diet 6C by lettuce and celery cabbage, and diet 6CC by celery cabbage only. The present paper concerns the effects of these diets on the reproduction and growth of offspring of rats.

METHOD OF STUDY

First, the effect of diet 6 on the reproduction and growth of offspring of 3 generations of Wistar rats was determined. After that, the effect of diets 6C and 6CC on 3 generations of Wistar and Sprague-Dawley rats was determined. In these tests, the rats were inbred by brother-sister matings so far as possible. In general 2 females were bred with each male. The aim was to obtain 3 litters from each fertile female on the vegetarian diets and to use only members of second or third litters for continuing the lines. However, in some cases members of first litters were used for further breeding because no members of later litters were raised. Moreover, F_2 generation females were mated with F_1 males when F_2 males of the

[1] This study was aided by a grant from Swift & Co., Chicago.

same strain seemed to be sterile, and finally F_2 Wistar females were mated with Sprague-Dawley males because all of the Wistar males that were reserved for breeding died. Litters of more than 8 young were reduced to 8 a few days after the litters were cast. After 3 litters were obtained on the vegetarian diet or the rats appeared to be sterile, they were fed an omnivorous diet (generally our basal omnivorous diet 1 ['47]), and its effect on the reproduction and growth of offspring was determined.

RESULTS

Data concerning the reproductive performance of the rats and the weights of their offspring at 42 days are presented in table 1. For comparison purposes, data are included in the table for rats fed the vegetarian diets, and for breeding rats fed our omnivorous diets. Data obtained on the 2 strains of

TABLE 1

Reproductive performance of rats on omnivorous and vegetarian diets and weights of offspring at 42 days.

GENERATION [1]	MALES MATED		FEMALES MATED		LITTERS CAST	INDIVIDUALS RAISED	MALES RAISED		FEMALES RAISED	
	Fertile		Fertile				Av. wt. at 42 days		Av. wt. at 42 days	
	no.	%	no.	%	no.	%	no.	gm	no.	gm
Rats on omnivorous diet										
F_0 to F_3	14	64	28	68	38	61	92	161	112	134
Rats on vegetarian diets										
F_0	9	89	18	94	49	21	39	66	43	62
F_1	8	75	14	100	37	13	19	50	17	47
$F_1♂ \times F_1♀$	2	100 [2]	4	100	4	19	3	44	2	39
F_2	4	50	6	50	3	0				
Rats on omnivorous diet after vegetarian diet										
F_0 to F_2 [3]	12	83	19	95	22	71	63	155	60	130

[1] F_0 generation omnivores means rats previously on a stock diet. F_0 generation vegetarians means offspring of rats fed omnivorous diets.

[2] Previously known to be fertile.

[3] Generations of vegetarians.

rats with the differently supplemented vegetarian diets are combined as no significant differences in the results were noted. As indicated, most of the rats on the vegetarian diets were fertile but they raised less than half the percentage of offspring raised by the rats fed our omnivorous diets, and these offspring of the vegetarians weighed less than half as much as those of the omnivores. Moreover, these young of the vegetarians weighed less in successive generations while those of the omnivores tended to be of equal weight. After the

TABLE 2

Season of the year in relation to type of diet, number of offspring born, and per cent of young raised.

DIET AND OFFSPRING BORN	SEASON OF YEAR			
	Dec.–Feb.	Mar.–April	May–Aug.	Sept.–Nov.
Omnivorous:				
Number born [1]	69	71	86	116
% raised	41	52	83	59
Vegetarian:				
Number born [1]	187	171	178	197
% raised	0	17	37	17
Omnivorous [2]*:*				
Number born [1]	47	57	46	22
% raised	74	56	85	73

[1] These are the number of rats born less the number sacrificed to reduce the size of litters of more than 8 new-born.

[2] After vegetarian diet.

vegetarians were fed one of our omnivorous diets, they raised a higher percentage of young than did the rats that were kept on our omnivorous diets, and their offspring immediately approached the weights of those of the omnivores. In fact, the male rat that became heaviest at 42 days (208 gm) was an offspring of ex-vegetarians.

Table 2 shows that the rats on the vegetarian diets raised no offspring in winter (3 winters included) and only 37% in summer. The omnivorous controls revealed a parallel but less extreme seasonal variation in the percentage of offspring

raised, but with respect to the vegetarians that were finally fed an omnivorous diet no significant seasonal influence on the percentage of offspring raised was observed.

Some of the offspring of the vegetarians were deformed at an early age but the deformities were evidently mainly due to the development of severe rickets. The inclusion of celery cabbage as a source of calcium (as well as of supplementary protein in the diet) did not prevent the development of severe rickets in some cases. At autopsy, enlarged joints and bowed fibula or tibia were found and the femurs of over half of the offspring of the vegetarians could not be removed without a separation of the head of the femur, due to poor calcification. This occurred even in offspring of the vegetarians that were over 200 days old and that had been fed our basal omnivorous diet (which included 3% bone meal) 30 days or more. Many of the offspring of the vegetarians were also more hyperexcitable and manifested more fear of being handled than did the offspring of rats on omnivorous diets, and the hypersensitivity did not decrease after the vegetarians were placed on an omnivorous diet. The sexual development of the vegetarians was retarded, and more so in the males than in the females. The feces of the vegetarians were often poorly formed.

DISCUSSION

The low percentage of offspring raised by the rats on the vegetarian diets was due to a high neonatal mortality and the apparent killing and eating of a large percentage of the young by the mothers. The killing of a pup by a vegetarian mother (by biting into the nape of the neck) was directly observed only once, but as many as 7 otherwise seemingly viable young were eaten by the vegetarian mothers during a single night. Occasionally, rats fed diets including meat also kill and eat their offspring (as observed) but the eating of a much larger percentage of offspring by our vegetarians was most likely due to the deficiencies in the vegetarian diets of suitable protein, calcium and other nutritional essentials. It is conceivable that the normal eating of the placentas after the

young are born whets the appetite of rats in need of such food and leads them to eat more than merely the placentas.

The failure of the vegetarians receiving celery cabbage to raise more offspring or heavier offspring than did the vegetarians receiving mainly lettuce as a fresh leafy vegetable supplement to the diet may be explainable by the relatively small amount of celery cabbage eaten. The rats ate much more lettuce than celery cabbage when both were supplied and they ate only slightly more celery cabbage when no lettuce was supplied. Some rats ate practically no celery cabbage, and the third generation of rats that were fed celery cabbage as the sole fresh leafy vegetable supplement to the diet still preferred lettuce when this was finally supplied. The lettuce fed by us consisted mainly of trimmings of head-lettuce (the green outer leaves and cuttings of the stems). The rats preferred this to leaf-lettuce, spinach, cabbage, celery, broccoli, cauliflower trimmings, carrot tops and beet tops. Variable amounts of greens other than lettuce were nevertheless usually also eaten when supplied with lettuce.

Considerable individual differences in the preferences or acceptances of the different items included in the basal part of the vegetarian self-selection diets were manifested by the rats, and changes in preferences occurred with ageing, pregnancy and in successive generations. The general order of preferences or acceptances of the different items was, however, approximately as follows: the germ part of kernels of corn, sunflower seeds, peanuts, green peas, the end of kernels of corn opposite the germ, defatted corn germ meal, barley, wheat, rolled oats, alfalfa leaf meal, soy beans, brewers' yeast, defatted wheat germ meal, salt (NaCl). The relatively low acceptance value of soy beans may help to explain why soy beans have not been used to any great extent even in China (according to Adolph, '44). Of special interest seemed to be the gnawing of the end of kernels of corn opposite the germ, mainly by young rats in the F_1 and F_2 generations. This end of the kernels of corn forms the surface of the ears and, as a result of pervaporation in the drying of the ears, the

surface evidently contains a concentration of water-soluble and presumably tasty components (Alexander, '33). The preference of the apparently more tasty part of the kernels of corn to the germ part suggests a finicky appetite. However, a sharp change to a preference of the germ part occurred in the females when they became pregnant. The general preference for the germ part of corn, sunflower seeds and peanuts to other items on the diet incidentally suggests that the fat or oil in these foods partly explains their appeal to the rats.

The period between weaning (at 35 days) and sexual maturity (as late as 150 days in some vegetarians) appeared to be the most difficult for the offspring of the vegetarians to bridge. About half of the males died during this period. The females fared better. Under more natural environmental conditions than those under which rats are kept in the laboratory they would evidently thrive better on an otherwise purely vegetarian diet as they would be able to resort to geophagy, osteophagy, the eating of insects and the eating of all dead rats. We removed dead rats, partly eaten rats and dying rats to prevent their being eaten. There was no evidence, however, of any tendency of adult vegetarians to resort to coprophagy as shown by rats on some vitamin deficient diets.

In contrast to the results of our study, Wu and Chen ('29) using a simple vegetarian diet supplemented alternately by colza and (Chinese) "small cabbage," found that their rats raised as high a percentage of offspring in the first 3 generations as rats fed their omnivorous stock diet. Their strain of rats may have been better adapted to living on a purely vegetarian diet than the strains we used, but it seems more likely that prolonged human experience of living on practically vegetarian diets in China has led to the cultivation of vegetables like colza and "small cabbage" which serve better as supplements to grains and legumes than leafy vegetables commonly raised in the United States. Moreover, the soil and agricultural methods used in China may serve to produce foods (grains and legumes as well as leafy vegetables) of

higher biological value than similar foods grown in the United States. Wu and his associates eventually raised 25 (or more) generations of rats with their vegetarian diet but the growth of the rats was subnormal and, after the first generation, 70% developed cataracts (Chen, Chang and Luo, '41). A purely vegetarian diet satisfactory for albino rats therefore still remains to be found.

SUMMARY

Rats on vegetarian self-selection diets including corn (whole kernels), wheat (whole grains), pearled barley, rolled oats, sunflower seeds, peanuts, soy beans, green peas, corn germ meal, wheat germ meal, brewers' yeast, alfalfa leaf meal, salt (NaCl), lettuce and celery cabbage were found to be generally fertile but raised less than 25% of their offspring. They raised none in winter. The growth of these young was also subnormal and severe rickets commonly developed.

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RELATION OF CARBOHYDRATE TO INTESTINAL SYNTHESIS OF BIOTIN AND HATCHABILITY IN MATURE FOWL[1,2]

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FIVE FIGURES

(Received for publication August 18, 1947)

It is well-recognized that changes in the kind of dietary carbohydrate may be reflected in the amount of vitamins synthesized by intestinal microorganisms (Guerrant, Dutcher and Tomey, '35; Morgan, Cook and Davison, '38; Mannering, Orsini and Elvehjem, '44; Najjar and Barret, '45; and Teply, Krehl and Elvehjem, '47). Evidence has been presented which shows that biotin is synthesized in animals (McElroy and Jukes, '40; Wegner et al., '41; Nielsen et al., '42; Mitchell and Isbell, '42) and in humans (Gardner et al., '43, '45, '46; Oppel, '42); it is synthesized by the bacteria of the intestinal tract (Landy et al., '42; Thompson, '42; Burkholder and McVeigh, '42).

The laying hen appeared to be a desirable experimental animal for studying intestinal synthesis since the vitamin content of the egg could be determined and would be a measure of the biotin available for metabolism. The use of the egg

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Supported in part by a grant from the Western Condensing Co., San Francisco, Calif.

[3] General Education Board Fellow.

as a measure of intestinal synthesis eliminates certain difficulties associated with collecting and assaying urine and feces in the chicken. Cravens, Sebesta, Halpin and Hart ('42) reported that biotin was necessary for normal embryonic development in the domestic fowl.

The present investigation was initiated to determine the effect of different carbohydrates on intestinal synthesis of biotin in the mature fowl. Sucrose, dextrin, and combinations of sucrose with lactose or dried whey were tested. Egg production, hatchability of fertile eggs, and biotin content of the eggs were the criteria used in evaluating the extent of intestinal synthesis and absorption of biotin.

MATERIALS AND METHODS

Twenty-four Single Comb White Leghorn pullets were placed in individual laying cages with raised screen bottoms, after being reared on the Experiment Station range. All birds were artificially inseminated weekly with mixed semen from New Hampshire cockerels. All eggs laid were marked with the hen's number and the date and settings were made weekly. The eggs were candled on the sixth and seventeenth days of incubation, at which times the infertile eggs and dead embryos were removed. All eggs which were removed on candling, and also those which failed to hatch, were broken and the age of the embryo at the time of death was estimated. Any gross abnormalities were recorded.

The pullets were fed an all-mash laying ration for 4 weeks prior to the start of the experiment, during which period data were collected on egg production and hatchability. At the end of this pre-experimental period the 24 birds were divided into 6 groups of 4 pullets each. At the end of the ninth week of the experimental period, the pullets which had been receiving diets B33 and B35 were placed on the practical all-mash diet and received the latter during the final week of the experiment. This change was necessitated by the poor condition of the birds at the end of the ninth week.

The materials used in this study were sucrose, dextrin (prepared by autoclaving moist cornstarch 2 hours with subsequent drying and grinding), lactose and dried whey. The composition [4] of the diets is shown in table 1. Crystalline B-vitamins were added in amounts which were thought to meet

TABLE 1

Composition of diets.

	B31	B32	B33	B34	B35
Sucrose — %	63		48	63	38
Dextrin — %		63			
Purified casein — %	18	18	18	18	18
Gelatin — %	5	5	5	5	5
Salts IV — %	5	5	5	5	5
Liver fraction "L" — %	4	4	4	4	4
Fish oil (3000 A) (400 D) — %	2	2	2	2	2
Soybean oil — %	3	3	3	3	3
Lactose — %			15		
Dried whey — %					25
Choline — %	0.2	0.2	0.2	0.2	0.2
Oyster shell			Ad libitum		
All vitamins listed below added as indicated in mg per kg of ration					
Biotin				0.2	
Thiamine HCl	4	4	4	4	4
Riboflavin	6	6	6	6	6
Ca pantothenate	15	15	15	15	15
Niacin	100	100	100	100	100
2-methyl-1,4-naphthoquinone	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	4	4	4	4	4
Alpha tocopherol	3	3	3	3	3

the requirements of the pullets with the exception of biotin and pteroylglutamic acid. Liver fraction "L" was used as a source of the latter vitamin. Biotin was omitted from all diets except B34 since this vitamin was the one under study. A practical all-mash diet was used as a positive control in addition to diet B34. Oyster shell and tap water were supplied ad libitum.

[4] We are indebted to the Western Condensing Co., for the lactose and dried whey; to Wilson Laboratories, Chicago, Illinois, for the liver fraction "L"; to Merck and Co., Rahway, N. J., for the biotin.

Eggs which were to be assayed for biotin were set aside on the last 2 days of each week. The microbiological method of Luckey, Moore and Elvehjem ('46), with slight modification, was used. The egg whites and egg yolks were separated and those from each group of birds were pooled for assay. After thorough mixing, approximately 10 gm were weighed out. An equal number of milliliters of 4N sulfuric acid was added and the sample autoclaved for 2 hours at 15 pounds pressure. In preliminary assays on eggs it was determined that hydrolysis with 4N, 8N or 12N sulfuric acid, as outlined above, gave approximately the same results. The 4N acid was selected since the sample would contain sodium sulfate on neutralization. After autoclaving, the pH was adjusted to 4–5 with 4N sodium hydroxide and the samples were filtered through Whatman no. 1 filter paper. The filtrate was neutralized to pH 6.8–7.0 and preserved under toluene in the refrigerator. Dilutions were made as needed. The organism used for the assay was Streptococcus faecalis R. The extent of growth of the organism was measured by turbidity using the Evelyn colorimeter. Satisfactory recoveries of biotin were obtained when known amounts of the vitamin were added to the samples. The biotin content of the egg whites and yolks is expressed in millimicrograms per gram on a fresh weight basis.

EXPERIMENTAL RESULTS

Egg production. The production of pullets fed the various diets is shown in figure 1. The egg production of pullets fed diets B31 and B32 and those fed the practical all-mash diet followed the same general trend throughout the 10-week period although a slight drop in the egg production of pullets fed sucrose, diet B31, may be noted during the last week of the experiment. The egg production of pullets fed sucrose plus biotin, diet B34, was somewhat erratic. The fluctuations in this case were traceable to 1 pullet and are more apparent due to the small number of pullets in the group; this pullet would lay a clutch of eggs and go out of production, then come back in production and repeat the same procedure.

The rather high levels of lactose and dried whey appeared to cause a decrease in egg production. The production of pullets fed diets B33, lactose, and B35, dried whey, decreased during the third and fourth weeks of the experimental period and remained at a fairly low level for the rest of the 10-week period. This low production was unfortunate because an insufficient number of eggs were laid to obtain a measure of

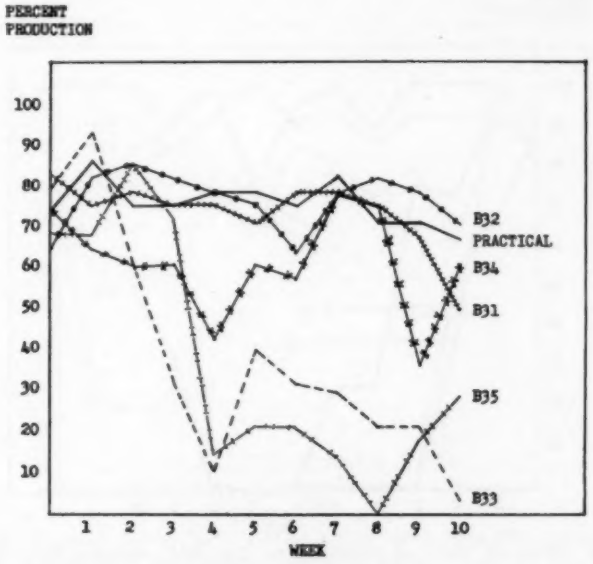

Fig. 1 Effect of diet on egg production: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

hatchability and for biotin assay in the case of pullets fed dried whey (diet B35).

Hatchability. The per cent hatchability of fertile eggs is shown in figure 2. Hatchability of eggs from pullets fed diet B31, sucrose, decreased to zero by the third week and remained at this level. Hatchability of eggs from pullets fed diet B32, dextrin, ranged from 60 to 85% during the 10-week period. This was somewhat lower than that of the positive control

groups which were fed diet B34 and the practical all-mash diet. Hatchability of eggs from pullets fed diet B34, sucrose plus biotin, was approximately the same as that of those fed the practical all-mash diet. The results with respect to hatchability obtained with diets B33, lactose, and B35, dried whey, were somewhat inconclusive after the third and fourth weeks due to the poor egg production which resulted from the

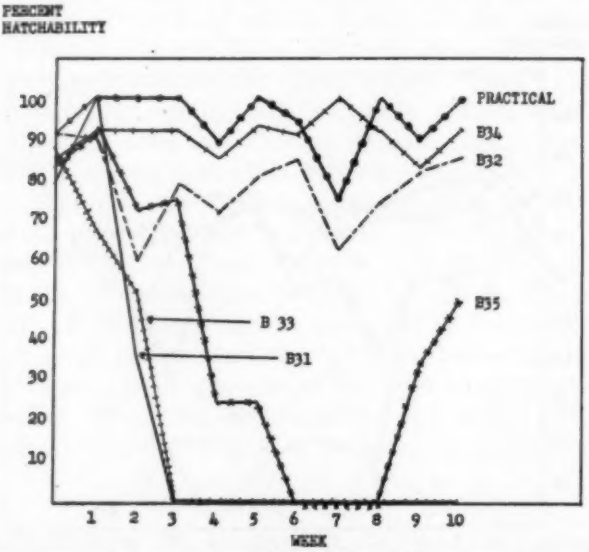

Fig. 2 Effect of diets on hatchability: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

amounts of these materials used in this study. This was discussed above.

Biotin content of egg white and yolks. At the beginning of the experiment the biotin content of the egg whites (fig. 3) varied from 65 to 105 mμg per gram expressed on a fresh weight basis. The biotin content of egg whites from hens fed diet B31 decreased from 65 to 17 mμg per gram during the first week and the presence of the vitamin was not detected in egg whites from these birds after this time. The level of biotin in

the whites of eggs from pullets fed diet B32 decreased from 88 to 22 mμg per gram during the first week and remained at the latter figure until the end of the third week; during the fourth week there was a further decrease from 22 to about 4–5 mμg of biotin per gram and it remained at this level through the eighth week.

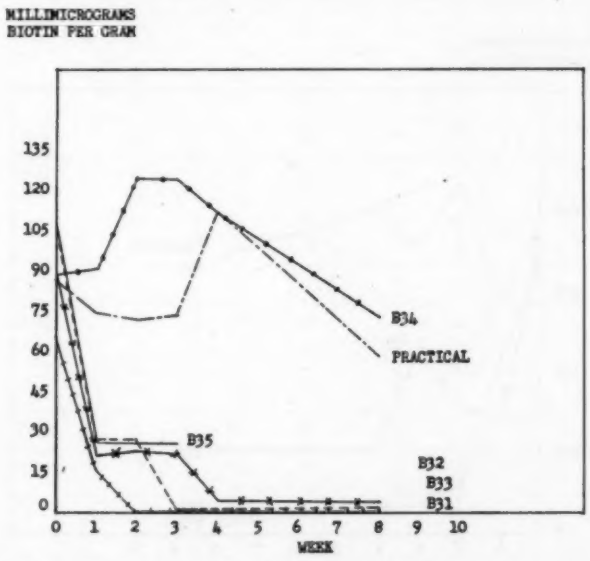

Fig. 3 Effect of diet on biotin content of egg white: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

The biotin content of egg whites from pullets fed diet B33 decreased from 105 to 27 mμg per gram during the first week and remained at the latter figure throughout the second week. At the end of the third week and thereafter, biotin could not be detected in egg whites from pullets fed diet B33. There was a decrease in the biotin content of egg whites from pullets fed diet B35 from 105 to 26 mμg of biotin per gram during the first week and it remained at the latter figure until the end of the third week. No eggs were analyzed from

pullets fed diet B35 after the third week due to the poor egg production of the birds. The feeding of 200 μg of biotin with sucrose, diet B34, was sufficient to maintain a slightly higher level of the vitamin in egg whites than was maintained in egg whites of pullets fed the practical all-mash diet (fig. 3).

The egg yolks from the pullets used in this study contained approximately 500 mμg of biotin per gram at the start of the

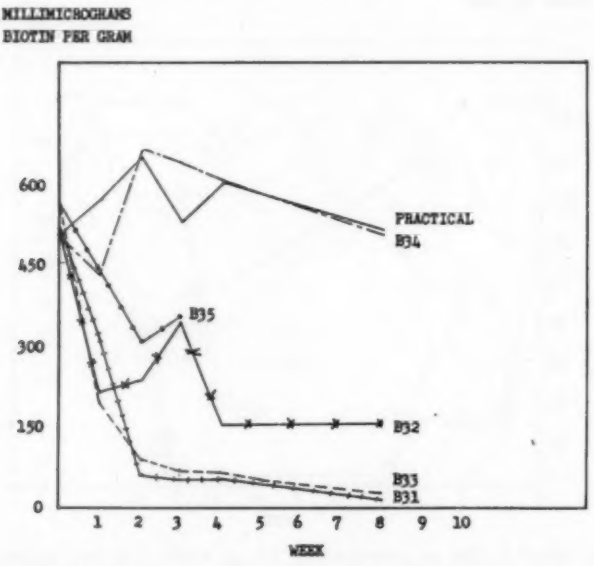

Fig. 4 Effect of diet on biotin content of egg yolk: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

experiment (fig. 4). The biotin content of the yolks from pullets fed diets B31, sucrose, and B33, lactose, decreased from about 500 to 50–70 mμg of biotin per gram during the first 2 weeks of the experiment. The egg yolks from pullets fed diet B32, dextrin, showed a decrease of from 500 to approximately 150 mμg of the vitamin per gram and remained at the lower level. The biotin content of egg yolks from pullets fed diet B35 followed the same general trend as those

TABLE 2

Effect of diet on incidence of syndactyly and other skeletal deformities.

HEN	DIET	NO. OF FERTILE EGGS	EMBRYOS DYING		NUMBER OF EMBRYOS SHOWING			
			Before 8 days	After 8 days	Beak parrot	Crooked tibia	Short twisted tarso metatarsus	SYNDACTYLY
1	B-31	49	19	18	8	7	7	7
2		13	3	7	3	6	6	6
3		16	14	0	0	0	0	0
4		35	18	14	8	2	2	5
	Total	113	54	39	19	15	15	18
5	B-32	40	2	2	0	0	0	0
6		48	2	6	0	0	0	0
7		49	2	4	0	0	0	0
8		43	7	9	0	0	0	0
	Total	180	13	21	0	0	0	0
9	B-33	28	9	11	3	5	5	4
10		13	7	1	0	0	0	0
11		11	1	4	4	4	4	3
12		9	1	7	1	2	2	4
	Total	61	18	23	8	11	11	11
13	B-34	37	2	3	0	0	0	0
14		42	1	0	0	0	0	0
15		33	0	4	0	0	0	0
16		6	0	0	0	0	0	0
	Total	118	3	7	0	0	0	0
17	B-35	26	10	4	0	2	2	1
18		15	1	0	0	0	0	0
19		6	0	0	0	0	0	0
20		8	1	1	0	0	0	0
	Total	55	12	5	0	2	2	1
21	Practical	42	1	2	0	0	0	0
22	Control	39	0	1	0	0	0	0
23	Diet	49	0	2	0	0	0	0
24		37	0	3	0	0	0	0
	Total	177	1	8	0	0	0	0

from pullets fed diet B32. Diet B34 and the practical all-mash diet supported about the same concentration of the vitamin in the egg yolks.

Embryonic mortality. The classification of the embryos that died during the incubation period is shown in table 2. There was a high incidence of micromelia and syndactyly in dead embryos from pullets fed diet B31. The micromelic embryos

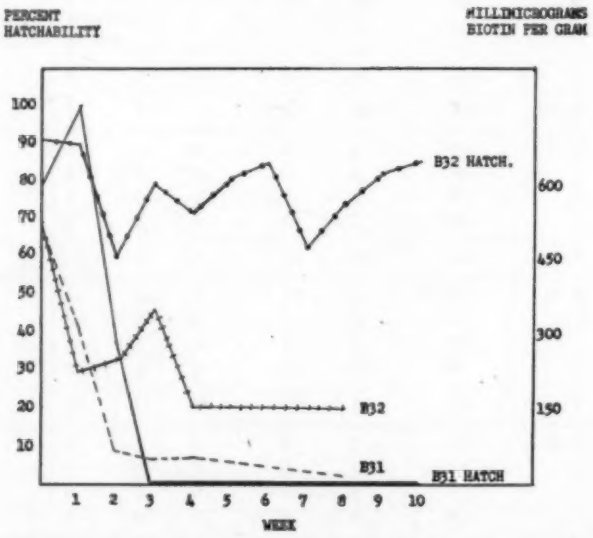

Fig. 5 Relation of carbohydrate to biotin content of egg yolk and hatchability: diet B31, sucrose; B32, dextrin.

were characterized by a parrot beak which was generally associated with a crooked tibia and a short twisted tarsometatarsus. Syndactyly and micromelia were not observed in dead embryos from pullets fed diet B32, diet B34 or from those fed the practical all-mash diet. The incidence of micromelia and syndactyly was high in the dead embryos from pullets fed diet B33 and was low in those from pullets fed diet B35, although these results are inconclusive due to the poor egg production of the birds.

DISCUSSION OF RESULTS

It may be noted from figures 2, 3, 4 and 5 that dextrin (diet B32) maintains a higher level of biotin in the egg yolk than does sucrose (diet B31) and that this is associated with a much higher per cent hatchability. The biotin content of egg yolks from pullets fed diet B31 decreased from about 500 to 50 mμg of biotin per gram during the first 3 weeks and had decreased to about 16 mμg of the vitamin per gram by the end of the eighth week. The decrease in the per cent hatchability of eggs from pullets fed diet B31 paralleled the decrease in biotin content of the yolk (fig. 5). The per cent hatchability was 79 at the start of the experiment. This increased to 100% for some unknown reason during the first week and decreased to zero by the end of the third week. It is apparent therefore that there was an insufficient amount of biotin to support embryonic development in the eggs from pullets fed diet B31. Thus it is indicated that eggs containing 50 mμg of biotin per gram of yolk and an undetectable amount in the white will not support normal embryonic development. In the case of pullets fed diet B32 there was a decrease in the biotin content of the yolk to about 150 mμg by the end of the fourth week and this amount of biotin was found in the yolks at the end of the eighth week. Hatchability of eggs from pullets fed diet B32 was 91% at the start of the experiment and varied from 60 to 85% in the course of the 10-week period. It is realized that the per cent hatchability of eggs from pullets fed diet B32 is lower than that of pullets fed diet B34 and also lower than that of pullets fed the practical all-mash diet. Embryonic abnormalities which appeared to be identical with those previously described by Cravens et al. ('44), as occurring in eggs from hens fed a low-biotin diet, were noted in eggs from pullets fed diet B31 but did not appear in eggs of pullets fed diet B32 (table 2). From these data it is indicated that eggs which contain 150 mμg of biotin per gram of yolk on a fresh weight basis will support embryonic development even though hatchability may be somewhat reduced.

Supplementation of the sucrose diet with biotin (diet B34) resulted in a per cent hatchability and biotin content of the eggs comparable to those obtained by feeding a practical all-mash diet (figs. 2, 3 and 4). These data indicate that the deficiency which existed in the pullets fed diet B31 was a biotin deficiency.

The results obtained with dextrin (diet B32) as compared to those obtained with sucrose (diet B31) suggest that there is a marked synthesis of biotin in the intestinal tract. Further, there is good evidence that the biotin synthesized in the intestinal tract of the pullets fed dextrin was absorbed and deposited in the egg. This latter fact was established through biotin analyses of the eggs and by the fact that the biotin in the eggs was available to the embryo as evidenced by the per cent hatchability.

This is in agreement with the work of Mannering et al. ('44) in which it was reported that dextrin favored the synthesis of riboflavin in the rat, and also with the work of Teply et al. ('47) in which it was reported that dextrin diets produced the greatest synthesis of niacin and folic acid in the rat.

The data presented in this paper indicate that the laying hen is a desirable animal for studying intestinal synthesis since a determination of vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult with other animals.

The studies reported herein shed no light on the important problem of where synthesis and absorption take place in the intestinal tract. Studies by Johansson et al. ('47) which were conducted on the birds used in the present investigation indicate that there was a marked difference in the intestinal flora of birds fed the various diets. Dextrin appeared to favor a biotin-synthesizing organism while sucrose favored a biotin-utilizing organism.

The biotin content of the eggs did not appear to be related to the egg production of the hens in the cases of pullets fed

diets B31 (sucrose) or B32 (dextrin). This is in agreement with the work of Cravens et al. ('42).

Results obtained by replacing a part of the sucrose with lactose (diet B33) or with dried whey (diet B35) are somewhat inconclusive due to the poor egg production of the pullets fed these diets. It is probably true that the level of lactose and dried whey used in these diets was too high for best results and suggests that the laying pullet may not tolerate lactose in quantities equal to that of the rat (Geyer et al., '46). Lactose, in the quantity used in diet B33, did not appear to favor the intestinal synthesis of biotin (figs. 2, 3 and 4). The biotin content and per cent hatchability of eggs from pullets fed diet B33 (lactose) followed the same general trend as those of pullets fed diet B31 (sucrose). Embryonic abnormalities, which were associated with a low biotin diet, were also observed in embryos from pullets fed diet B33 (table 2).

The results obtained with diet B35 (dried whey) were quite inconclusive after the third or fourth week due to the poor egg production of the pullets fed this diet (fig. 1). There was a higher level of biotin in the egg yolks and whites and a higher per cent hatchability of eggs from pullets fed diet B35 during the first 3 weeks than was noted in eggs from pullets fed diets B31 and B33. This may have been traceable to the fact that 25% dried whey contributed approximately 40 μg of biotin per kilogram of diet. A small number of embryos exhibiting symptoms of a biotin deficiency were noted in the eggs of 1 pullet fed this diet (table 2).

The term "micromelia" is used in contrast to the term "chondrodystrophy" in referring to the characteristic embryonic abnormality associated with a deficiency of biotin in the diet of the mother hen. It is recognized that the term "chondrodystrophy" designates a certain histopathologic picture. It should be pointed out, however, that from a gross macroscopic study the micromelia embryos recorded in table 2 of the present report and described by Cravens et al. ('44) are indistinguishable from the chondrodystrophy which has been shown to have a hereditary basis (Lamoreaux, '42).

The occurrence of micromelia and syndactyly in embryos from pullets fed diet B31 extends and confirms the previous work of Cravens et al. ('44). It may be noted from table 2 that the characteristics which describe micromelia: parrot beak, crooked tibia and short-twisted tarsometatarsus appeared to occur in about the same frequency as syndactyly. This indicates that syndactyly is a definite symptom of biotin deficiency in the developing chick embryo as was also observed by Cravens et al. ('44). Micromelia and syndactyly were observed among embryos which lived beyond the eighth day of incubation from pullets fed diets B31 and B33. The fact that eggs from these birds were low in biotin has been discussed earlier. The incidence of micromelia and syndactyly appeared to be fairly well distributed among the individual pullets fed diets B31 and B33 (table 2).

SUMMARY

Evidence is presented to show that dextrin favors the intestinal synthesis of biotin in the mature fowl and sucrose does not promote the synthesis of this vitamin. The results obtained with lactose and dried whey indicate that lactose does not stimulate the synthesis of biotin in the intestinal tract of the laying pullet.

The laying hen is a desirable animal for studying intestinal synthesis. A determination of the vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult in other animals.

There is a failure of embryonic development when the egg yolk contains as little as 50 mμg of biotin per gram on a fresh weight basis. Eggs which contain 150 mμg of biotin per gram will support normal embryonic development.

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HUMAN UTILIZATION OF ASCORBIC ACID FROM MUSTARD GREENS

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TWO FIGURES

(Received for publication September 26, 1947)

Previous investigations have shown that, for many families, cooked vegetables are the main dietary source of ascorbic acid (Grigsby et al., '42; Bryson et al., '42; Coco et al., '43; Whitehead, '47). In the far South cooked greens, repeatedly shown by chemical determination to be good sources of vitamin C, are commonly used. A study of the availability for human subjects of vitamin C from such sources is therefore of practical value to the nutritionist.

A number of studies have been reported which pertain to the utilization of dietary ascorbic acid as compared to that of the crystalline vitamin. The levels at which the vitamin has been fed, the number of subjects and the duration of the test periods have varied widely. Some workers have based conclusions on urinary excretion of the ascorbic acid, while others have used plasma or whole blood levels, and a few have used both plasma or whole blood levels and urinary excretion.

After a study of the urinary excretion of ascorbic acid following the administration of comparable quantities of this vitamin as orange juice and in the pure crystalline form, Hawley, Stephens and Anderson ('36) concluded that the vitamin C of orange juice was as well utilized as the pure compound.

Baked potato was found by Clayton and Folsom ('40) to produce as high plasma ascorbic acid values as did the crystalline vitamin. Two-thirds of the dietary ascorbic acid was supplied by this vegetable.

Todhunter and Fatzer ('40) compared the utilization of ascorbic acid from red raspberries with that of the crystalline vitamin. Blood plasma ascorbic acid levels and urinary excretion of ascorbic acid were similar for the same subject, regardless of the source of the vitamin.

In 1942 Todhunter, Robbins and McIntosh reported a study of the rate of increase of blood plasma ascorbic acid after ingestion of 50 mg of ascorbic acid in the crystalline form, and after consumption of equivalent amounts in cauliflower, orange sections, orange juice or strawberries. Orange juice and orange sections produced the maximum rise in plasma level as quickly as the pure substance, while strawberries required slightly longer, and cauliflower the longest time of all the foods tested.

Using the ability of the supplement to maintain tissue saturation and to support plasma ascorbic acid levels as the criteria for judging availability, Clayton and Borden ('43) concluded that both canned tomato juice and raw cabbage produced as good results as the vitamin in tablet form.

Hartzler ('45) found no significant differences in the availability of the ascorbic acid of papayas or guava juice as compared with synthetic ascorbic acid. The criteria used for comparison of availability were the daily urinary excretion and plasma ascorbic acid levels determined once each week.

Einbecker et al. ('47) found that increases in plasma ascorbic acid produced by a test meal including frozen strawberries were comparable to those obtained when crystalline vitamin C was taken in amounts equivalent to the reduced ascorbic acid in the strawberries. Increases obtained when crystalline ascorbic acid was taken in amounts equivalent to the apparent total ascorbic acid of the strawberries were greater than those obtained after the ingestion of strawberries.

Elliott and Schuck ('47) reported approximately equal utilization of ascorbic acid from grapefruit and from the crystalline vitamin. The criteria upon which this conclusion was based were urinary excretion of ascorbic acid by 9 subjects during two 3-day test periods and whole blood ascorbic acid levels of 3 subjects at the end of each test period.

EXPERIMENTAL PROCEDURES

Mustard greens were chosen as the green leafy vegetable for this experiment because they are widely used in the South and a good supply was available at a convenient time.

The daily basal diet consisted of 1 egg, 1 pint of pasteurized milk, and 1 serving of each of the following: a dried fruit, a canned fruit, thoroughly cooked meat, canned snap beans, and a cooked fresh vegetable. Carrots, eggplant, potatoes, and mature dry onions were the vegetables used. The eggplant was sautéed, while the other fresh vegetables were boiled in a large amount of water to reduce the ascorbic acid content. The potatoes were always mashed. Whole grain and enriched cereals, bread, cake, cookies, and baked beans were used *ad libitum*.

During the periods when the mustard greens were eaten, the greens were substituted for the canned snap beans in the noon meal. During the periods when the synthetic vitamin was used, the ascorbic acid supplement was also given at the noon meal. Since the diet was believed to be adequate in all respects except ascorbic acid, no other vitamin supplements were given. The mustard greens were blanched and frozen before the beginning of each experiment to insure a regular supply. A pressure saucepan was used for cooking as previous experience (Hollinger, '44; Hollinger and Colvin, '45) had shown that this method of cooking results in maximum retention of vitamin C. The greens were seasoned with sufficient salt and bacon fat before cooking to make them palatable.

In 1945, 5 young women served as subjects. The plan of the experimental periods is given below.

Experimental periods — 1945

Period I, 14 days. Basal diet containing 13 mg of reduced ascorbic acid.

Period II, 21 days. Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap beans. The average ascorbic acid content of this diet was 57 mg, of which 49 mg were supplied by the greens.

Period III, 14 days. Basal diet as in Period I with supplement of ascorbic acid in water solution to give same average total intake as in Period II.

Immediately after breakfast blood samples were drawn by venous puncture on 5 consecutive days each week. Analyses were made immediately by the Mindlin and Butler ('38) macro method. The method was followed in detail except that no potassium cyanide was used.

All foods containing ascorbic acid were eaten in constant weighed amounts and a determination of the reduced ascorbic acid was made daily on a composite sample representing one-fourth of the intake. Determination of ascorbic acid in the food was made by the dye titration method.

In 1946 the plan of the experiment was altered in order to provide for a period of high ascorbic acid intake immediately preceding the beginning of the experimental period and again following the period when mustard greens were eaten. The plan of the experimental period is given below. This plan insured a more uniform state of nutrition among the subjects than is probable when no preparation of the subjects is carried out.

Experimental periods — 1946

Period I, 15 days. Uncontrolled diet. One hundred milligrams ascorbic acid daily in tablet form.

Period II, 14 days. Basal diet containing an average of 15 mg of ascorbic acid.

Period III, 15 days. Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap

beans. The ascorbic acid content of this diet was 57 mg, of which 49 were supplied by the cooked greens.

Period IV, 15 days. Same as Period I.

Period V, 14 days. Same as Period II.

Period VI, 15 days. Basal diet supplemented by sufficient ascorbic acid in solution to bring the average total intake of the vitamin to that in Period III.

Blood samples were drawn immediately after breakfast 4 times each week. The samples were taken on Monday, Tuesday, Thursday and Friday in order to compare values on consecutive days. Eight samples from each subject were analyzed during the depletion periods (Periods II and IV) and 10 during the periods when the greens and the ascorbic acid were given (Periods III and VI).

Three young men and 4 young women served as subjects for this portion of the study. Three of the young women (subjects J.G., L.J., and V.W.) had served as subjects in 1945.

RESULTS

In 1945 the average plasma ascorbic acid values for 3 subjects were almost the same on both regimes. For 1 subject the average plasma ascorbic acid value was 14% higher on the ascorbic acid regime than on the mustard green regime, while in another the crystalline vitamin produced an average plasma ascorbic acid value 33% higher than that produced by mustard greens (table 1). In 1946, 6 subjects showed slight increases in plasma ascorbic acid values during the mustard green regime when these values were compared to the average for the 2 days just previous. One subject had the same average plasma value (table 2). During the period when ascorbic acid was taken, 4 subjects showed gains while the remaining 3 showed slight losses. Subject V.W. showed considerably greater gain on crystalline ascorbic acid than on mustard greens. It should be noted that this subject had served in the 1945 study and at that time also attained higher plasma ascorbic acid values when receiving the pure vitamin. Subjects

J.G. and L.J. who also had served in the 1945 study showed in both experiments approximately the same plasma ascorbic acid values on the ascorbic acid regime as on the mustard greens diet.

TABLE 1

Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1945.

SUBJECT	SEX AND WT. IN KG	PERIOD I BASAL DIET [1]	PERIOD II MUSTARD GREENS [2]	PERIOD III ASCORBIC ACID [3]
D. C.	F. 57	0.50	0.46	0.48
J. G.	F. 68	0.67	0.53	0.53
L. J.	F. 66	0.30	0.48	0.51
H. L.	F. 57	0.45	0.58	0.66
V. W.	F. 50	0.58	0.58	0.76
Average		0.50	0.53	0.59

[1] Average of 2 days preceding mustard green period.
[2] Average of 15 determinations during 3 weeks.
[3] Average of 10 determinations during 2 weeks.

TABLE 2

Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1946.

SUBJECT	SEX AND WT. IN KG	AT END OF PERIOD II [1]	MUSTARD GREENS [2]	AT END OF PERIOD V [1]	CRYSTALLINE ASCORBIC ACID [2]
J. B.	M. 60	0.39	0.46	0.52	0.51
J. G.	F. 68	0.47	0.52	0.48	0.51
V. H.	F. 60	0.56	0.57	0.54	0.61
L. J.	F. 66	0.45	0.45	0.50	0.49
F. L.	M. 72	0.29	0.36	0.50	0.43
J. W.	M. 76	0.36	0.39	0.36	0.39
V. W.	F. 50	0.52	0.54	0.50	0.63
Average		0.44	0.47	0.48	0.51

[1] Average of plasma values for last 2 days of period.
[2] Average of plasma values (10 determinations during 2 weeks).

Higher plasma values were maintained at all times by the smaller subjects while the larger subjects showed a more rapid decline on the basal diet and a very slow decline on both the mustard green and the crystalline ascorbic acid regime.

This suggests that the average daily intake of ascorbic acid, 59 mg, was inadequate for the larger individuals. Typical curves are shown in figures 1 and 2. Plasma values for the men showed fluctuations similar to those for the women.

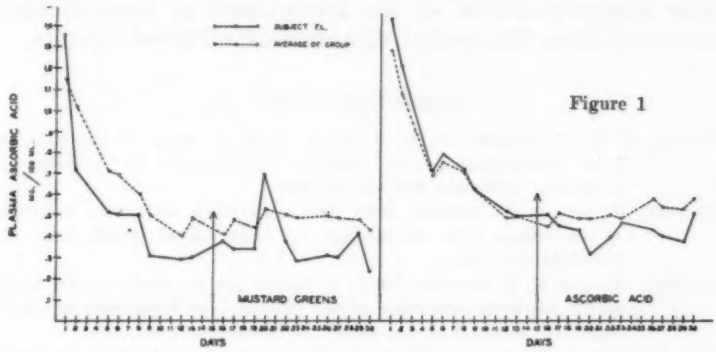

Figure 1

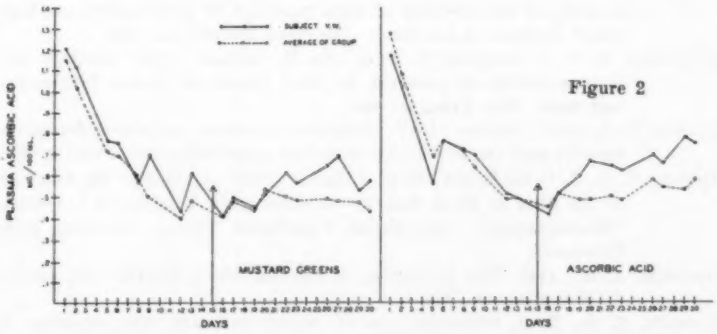

Figure 2

Figs. 1 and 2 Comparison of plasma ascorbic acid values of subjects F.L. and V.W. and average plasma ascorbic acid values of 7 individuals — 1946.

CONCLUSION

It is concluded that some individuals utilize the ascorbic acid from cooked mustard greens just as well as the synthetic vitamin. Other persons fail to show as high plasma ascorbic acid values when mustard greens are the main source of the vitamin as they do when the crystalline vitamin is given.

ACKNOWLEDGMENTS

This study was supported in part by funds provided by the General Education Board.

The author also wishes to acknowledge the assistance of Miss Margaret Green, of the Department of Bacteriology, Louisiana State University, who obtained all blood samples.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

III. LOW PROTEIN INTAKE [1]

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(Received for publication September 16, 1947)

Two recent publications in this journal by Forbes, Swift and associates ('46a, '46b) demonstrated that liberal amounts of fat in the diet of the growing rat resulted in a superior energy utilization of the diet as a whole. Increased body weight, comprising greater gains of fat and energy, and a decrease in the heat production of growing rats resulted as the fat content of isocaloric diets was increased from 2 to 30%. In the preparation of the diets used in these experiments an effort was made to furnish all nutrients in optimum quantities for the most efficient utilization of the food energy, including an excellent quality protein mixture furnishing 22% protein in the diet.

In view of the shortages of foodstuffs current in many areas of the world, especially of fat and protein, it seemed of further interest to investigate the effect of decreasing the dietary protein in similar high and low fat diets on the resulting energy utilization. It was decided, therefore, to follow the plan of the earlier experiments, namely, to use diets containing 2 and 30% of fat, but with a protein content of 7% in the low fat diet and an equivalent amount in the high fat diet.

[1] Authorized for publication on September 15, 1947, as paper no. 1391 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

EXPERIMENTAL

The experimental subjects were 12 pairs of litter-mate male albino rats of the Wistar strain selected immediately after weaning. Each rat of a pair was fed isocaloric amounts of one of the 2 diets for a period of 70 days, using the paired-feeding technique. Both rations supplied the same amount of protein and vitamin supplements, and differed inversely in their fat and carbohydrate contents.

The subjects were allowed normal cage activity. The heat production as determined by the body balance method of this laboratory (Swift et al., '34) provided a single measurement for the entire 70-day period through the subtraction of the energy of the excreta and of the body gain from the gross energy of the food.

Daily feces and urine collections were made according to the method referred to above and all energy values of the diets, bodies, and excreta were determined by the bomb calorimeter. Nitrogen and crude fat determinations were made according to the Kjeldahl and Soxhlet extraction procedures, respectively.

The 2 diets, prepared from purified foodstuffs in the manner described earlier, were analyzed before the start of the experiment and stored in the refrigerator. Tables 1 and 2 present the composition of the diets and the quantities of major nutrients supplied.

The average weekly live weights for the 12 rats on each of the 2 diets are shown in table 3. It is apparent that the animals on the high fat diet gained more weight than the low fat group as the experiment progressed. The statistical odds that this difference in weight gain is significant are 50:1. The difference in live weight is further emphasized, however, by the method of food assignment used which has the effect of placing at a disadvantage those animals that increase more rapidly in weight since a larger proportion of the food intake is then required for maintenance and less is available for growth.

The possibility that this increased weight gain could be in the form of water is contraindicated by the data on the body

TABLE 1

General composition of diets.

COMPONENTS	DIET 1	DIET 2
	%	%
Protein-containing mixture [1]	11.00	15.63
Carbohydrate mixture [2]	83.00	50.37
Corn oil [3]	2.00	2.00
Lard [4]	0.00	28.00
Salt mixture [5]	4.00	4.00
Cal. per gm	3.869	5.498
Isocaloric factors	1.000	0.704

[1] Casein 50%, skim milk powder 25%, irradiated yeast 25%. Mixture contained 60.5% protein (N × 6.25) and 4.71 cal. per gm.

[2] Corn starch 34%, sucrose 33%, dextrin 12%, and dextrose (cerelose) 21%. Mixture contained 3.75 cal. per gm.

[3] Mazola. Contained 9.52 cal. per gm.

[4] Contained 9.33 cal. per gm.

[5] U.S.P. XII. no. 2.

TABLE 2

Vitamins added per kg of diet 1 and to isocaloric quantities of diet 2.

VITAMINS		VITAMINS	
	mg		*mg*
Carotene	40	Choline chloride	2000
Thiamine hydrochloride	20	Alpha tocopherol	200
Riboflavin	20	P-aminobenzoic acid	200
Pyridoxine hydrochloride	20	2-methyl-1, 4 naphtho-	
Niacin	20	quinone	6
Calcium pantothenate	100	Inositol	2000

TABLE 3

Average live weights of rats during 10 weeks on isocaloric quantities of diets containing 2% and 30% of fat.

FAT CONTENT OF DIETS	INITIAL BODY WEIGHT	WEEK NUMBER									
		1	2	3	4	5	6	7	8	9	10
%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
2	54	58	70	81	89	97	105	113	123	129	135
30	54	58	71	83	91	99	107	116	126	133	140

gain of fat which are summarized in table 4. The rats on the high fat diet gained, on an average, 14% more energy in the form of fat than the group on the low fat diet. The statistical odds that this difference is not due to chance are 25:1.

The average gain of ether extract in the rats on the 2% fat diet was 19.4 gm, while their average intake was only 11.5 gm.

TABLE 4

Average food consumption during 70 days; and initial and final nitrogen and fat contents of rat bodies.

FAT CONTENT OF DIETS	FOOD CONSUMPTION	NITROGEN INTAKE	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	gm	gm	gm	gm	gm	gm
2	576.4	6.48	1.30	3.85	4.13	19.36
30	405.8	6.31	1.30	3.86	4.13	21.67

TABLE 5

Partition of average nitrogen intake per rat during 70 days.

FAT CONTENT OF DIETS	INTAKE OF N	OUTPUT OF N IN		N RETAINED [1]	N RETAINED [2]
		Urine	Feces		
%	gm	gm	gm	gm	gm
2	6.48	2.89	0.85	2.74	2.55
30	6.31	3.04	0.71	2.56	2.56

[1] Nitrogen in food minus nitrogen in the excreta.

[2] Nitrogen in bodies of rats fed for 70 days minus nitrogen in the bodies of the control group.

Therefore there was an average of nearly 8 gm of fat synthesized by the rats on this diet. The ether extraction procedure was employed to separate the body residue into 2 portions for the purpose of quantitative grinding and sampling. No effort was made to determine precisely the true fat gains since the weights of body fat were not of primary significance.

A summary of the nitrogen utilization data and the partition of the nitrogen intake are presented in table 5. The reduction in protein content of these diets in comparison with the earlier work resulted in a considerably decreased growth rate and nitrogen retention. However, the difference in fat

content of the diets was found to exert no significant influence on the nitrogen retention. This finding is in accord with that of the former work which indicated that the influence of large amounts of fat in the diet on the nitrogen utilization was not very definite.

The recovery of the feed nitrogen in the feces, urine, and body gain was, on the average, 97 and 100% of the intake for the 2% and 30% fat diets, respectively. The comparison of nitrogen retained as calculated from intake and excretion, and the nitrogen of the body gain were in close agreement.

From the data in table 6 it is evident that the difference in fat content of the diets resulted in a significant difference in heat production. The high fat animals produced 2.4% less

TABLE 6

Partition of average daily intake of food energy per rat during 70 days.

FAT CONTENT OF DIETS	GROSS ENERGY INTAKE	ENERGY INTAKE				ENERGY OUTPUT			ENERGY RETAINED
		Protein	Carbohydrate	Fat	Metabolizable	In feces	In urine	As heat	
%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
2	2230	233	1886	111	2132	62	37	1894	238
30	2230	226	837	1167	2112	88	31	1849	263

heat than the pair-mates on the low fat diet. The odds that this difference is not due to chance are 280:1.

Slightly more energy was excreted in the feces and less in the urine of the animals on the high fat diet as compared to the low fat group but the resulting metabolizable energy values were essentially the same.

In general, the effect of reducing the protein content of the diets from about 22%, as fed to growing rats in the previous work, to 7% in the present study was mainly in degree rather than in nature. Despite the lower protein content of the diets the high fat ration exerted a favorable effect upon the energy utilization, demonstrated by an increase in body gain and a decrease in heat production. It seems probable from this evidence that the level of dietary protein is not a factor in the

superior energy utilization of high fat diets exhibited by growing albino rats under conditions of normal cage activity.

SUMMARY

An investigation of the possible influence of low protein intake on the superior energy utilization of high fat diets was conducted using 12 litter-mate pairs of weanling male albino rats subjected to a 70-day metabolism and body analysis procedure.

A comparison was made between 2 diets containing 2% and 30% of fat, respectively, so prepared and fed as to supply to each rat of a litter-pair and therefore to each group of 12 the same quantities of energy, protein and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, and of the heat production for 70 days as the energy of the food minus the energy of the excreta and of the body gain.

Decreasing the protein intake from the former 22% level to 7% of the diet did not alter the previously reported superior energy utilization of high fat diets by the growing rat. In the present experiment an increased weight gain, increased body gains of fat and energy, and decreased heat production were associated with the high fat ration.

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EFFECT OF THE PTEROYLGLUTAMIC ACID INTAKE ON THE PERFORMANCE OF TURKEYS AND CHICKENS[1]

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(Received for publication August 21, 1947)

Previous work has shown that the young turkey requires a dietary source of pteroylglutamic acid (folic acid) and that a deficiency of this factor in the diet results in retarded growth, moderate anemia, cervical paralysis and a high mortality (Richardson et al., '45; Jukes et al., '47; Russell and Taylor, '47). Available data indicate that 0.8–2.0 mg of pteroylglutamic acid/kg of ration is required for the young poult (Jukes et al., '47; Russell and Taylor, '47).

In a recent report by Taylor ('47) data have been presented on the influence of the amount of pteroylglutamic acid (PGA) in the diet of the White Leghorn on egg production and hatchability. It was concluded that 0.12 mg/kg of PGA or less was required for high egg production but that more than this amount was needed for sustained hatchability. No work has been reported, however, on the effect of the amount of this vitamin in the diet on the performance of adult turkeys for egg production or hatchability.

In the present study the influence of the amount of PGA in the diet on the performance of adult turkeys and chickens and of young poults has been determined. Data have also been

[1] This work was supported in part by a grant from Lederle Laboratories Division, American Cyanamid Co. We wish to express our appreciation to Frances Panzer and Helen Keene for assisting with the analytical work.

obtained during the past 2 seasons on the amount of PGA found in the egg and in the blood of both chickens and turkeys as influenced by the dietary intake, the survival and performance of young poults obtained from groups of hens fed diets high and low in PGA, and egg production and hatchability. The results obtained in these experiments are reported in this paper.

EXPERIMENTAL

Care of experimental birds. Broad-breasted, bronze turkey hens that were 6 months of age were placed in breeding pens at an average initial weight of approximately 7.7 kg. Eight birds were used in each of 2 groups; 1 received a diet designed to be low in PGA and the other received the same diet to which 2.0 mg PGA/kg were added. Insofar as possible the birds were distributed uniformly between the 2 groups. The houses were lighted at all times and good egg production was achieved in a period of 2 weeks. The hens were then trapnested and records obtained on egg production. At uniform intervals throughout the experiment studies were also conducted on the hatchability of the eggs and on the amount of PGA in the egg. A similar allocation of experimental birds was used in each of the 2 seasons that this study was carried out.

First-year White Leghorn pullets (hereafter referred to as hens for simplicity) were used in parallel investigations. Fifteen hens were used in each group, 1 group receiving the basal ration and the other the PGA supplemented ration. In all cases matings were carried out with males maintained on a standard ration and insofar as possible the males were rotated among several groups of hens at regular intervals.

A series of studies was also carried out with young poults in which the effect was determined of the amount of PGA ingested by the hens on the survival and growth of the young poults fed diets deficient or adequate in the vitamin. In this work day-old poults both from the deficient and supplemented hens were used. Poults hatched from eggs laid by each hen were distributed uniformly among the experimental groups.

Composition of rations. The composition of the rations fed the adult turkeys is given in table 1. The basal ration was designed to be low in PGA and adequate insofar as possible in other nutrients. The control group was fed, in addition, 2.0 mg of PGA/kg of ration. The B vitamin supplements were prepared in the following manner. An aqueous suspension of the vitamins was added to finely ground corn, dried, reground to a fine powder and thoroughly mixed with additional ground corn so that 1% of the mixture supplied the correct amounts of the vitamins in the ration. The adult chickens were fed a similar ration, differing only in that 2% less casein was used with a corresponding increase in the amount of cerelose.

In preliminary tests conducted during 1946 a ration similar to that just described, except higher in protein, was used in studies with the young poult. In 1947, however, a purified ration of known PGA content was used. The effect of the diet of the hen on the performance of the young poult could, therefore, be more accurately evaluated. The composition of the purified ration used in this work with the young poults is also given in table 1. The amount of PGA added to the ration was varied, being 0.2, 0.8 or 2.0 mg/kg. These levels were used in order to determine the effect of feeding amounts either below, approximating or exceeding the reported requirement.

Methods of analysis. The pteroylglutamic acid content of the eggs was determined microbiologically with the use of *S. faecalis* R as the test organism (Teply and Elvehjem, '45). The vitamin was liberated from the samples by takadiastase treatment in a manner similar to that used for animal tissues (Schweigert et al., '46). The entire content of each egg was weighed and homogenized in a Waring blendor with an equal weight of water. An aliquot of the homogenate (equivalent to 5 gm of egg) was then diluted to approximately 75 ml, adjusted to pH 4.5 with HCl and sodium acetate, and incubated with 100 mg of takadiastase for 16–18 hours at 37°C. After autoclaving for 5 minutes to inactivate the enzymes, the samples were diluted to an appropriate volume, neutralized, filtered and aliquots taken for assay.

TABLE 1

Composition of rations.

RATIONS FOR ADULT TURKEYS				RATIONS FOR TURKEY POULTS			
	%		*mg/kg*		%		*mg/kg* [1]
Ground yellow corn	65.54	Thiamine	6.0	Casein	25	Thiamine	6.0
Casein	10	Riboflavin	6.6	Gelatin	10	Riboflavin	6.6
Raw bone meal	8	Pyridoxine	6.6	Corn oil	4.25	Pyridoxine	6.6
Cerelose	11.4	Ca pantothenate	22	Salts [2]	5.0	Ca pantothenate	22
Cellulose	2	Nicotinic acid	50	Fortified A and D oil	0.75	Nicotinic acid	50
Oyster shell	2			Cystine	0.2	Inositol	1000
NaCl	0.6	Pteroylglutamic		Starch	54.8	Choline	2000
$MnSo_4 \cdot 4H_2O$	0.013	acid	0 or 2.0			p-aminobenzoic acid	2.0
Choline	0.17					Biotin	0.2
Fortified A and D oil	0.25					2-methyl 1,4 naphthoquinone	20
Delsterol	0.026					Mixed tocopherols	100
						Pteroylglutamic acid	0.2, 0.8 or 2.0

[1] Wilkening and Schweigert ('47).

[2] Hegsted et al. ('41).

A series of determinations conducted during the 1946 season indicated that the amount of PGA added to the diet did affect the amount in the eggs. Therefore, during the present season, 8–15 eggs were taken from each group of chickens and turkeys at regular monthly intervals for PGA analysis.

At the conclusion of the feeding tests conducted with adult chickens and turkeys, samples of blood were drawn for determinations of free and combined PGA (Schweigert and Pearson, '47). The feed was withheld from the birds for 3 hours prior to taking of the blood samples. Six to 8 ml of blood were drawn from the turkeys from the wing vein and from the chickens by heart puncture into oxalated tubes. Determinations were made of the "apparent" free folic acid in whole blood and plasma, and in some cases of the amount present after enzymatic digestion on both plasma and whole blood.

RESULTS AND DISCUSSION

No effect attributable to feeding the diet low in PGA to adult turkeys or chickens was observed on general appearance, mortality, hemoglobin level, or weight change. Furthermore, no appreciable difference was noted in egg production and hatchability for the 2 species. The egg production for the group of 8 turkeys fed the diet low in PGA totalled 1108 and the egg production for the supplemented group totalled 1067 for the 2 seasons. Egg production for each group of 15 chickens fed the 2 corresponding diets was 1928 and 1945, respectively. Thus, it appears that sufficient PGA was present in the basal diet as judged by these criteria. By microbiological analysis, the basal ration was shown to contain 0.42 mg of PGA/kg of ration.[2]

It is significant to note that Taylor ('47) observed that 0.12 mg of PGA/kg was sufficient for satisfactory egg production and somewhat more was needed for hatchability. It would seem, therefore, that the requirement for good hatchability was between 0.12 and 0.42 mg of PGA/kg.

[2] We are indebted to Dr. T. H. Jukes and Dr. E. L. R. Stokstad for this analysis. A chick pancreas enzyme preparation was used to liberate the PGA.

The level of PGA in the diet does, however, markedly influence the amount of the vitamin present in the egg. Preliminary results were obtained on eggs from turkeys maintained for 5–6 months on the experimental regimen described and those from chickens maintained for periods of 1–2 months during the 1946 season. Twenty turkey eggs from the low PGA group averaged 0.113 μg of PGA/gm; 26 eggs from the PGA supplemented group averaged 0.195 μg of PGA/gm, while 13 eggs from hens fed the standard flock ration averaged 0.171 μg of PGA/gm. Separate studies showed that most of the vitamin was present in the egg yolk: 0.47 and 0.31 μg of PGA/gm of yolk for the supplemented and deficient groups, respectively; and 0.013 and 0.007 μg of PGA/gm of egg white, respectively.

Eggs from chickens fed the basal and supplemented diets for 1 month were identical in their PGA content: 0.067 and 0.064 μg/gm of whole egg; however, eggs obtained after 2 months of feeding averaged 0.12 and 0.20 μg of PGA/gm, respectively.

Experiments conducted during 1947 were designed to obtain eggs from each group at regular intervals from the beginning to 7½ months (table 2). Unfortunately, egg production was not at a high enough rate to merit analyses prior to 1½ months on experiment. It will be noted that a difference was obtained in the content of PGA in 1½ months of feeding. The difference in the amount found in the eggs did not appear to become greater during the test period. Thus the mean difference was as great at 2½ or 3½ months of feeding as it was at 6½ or 7½ months. Insofar as possible, determinations were made on eggs from each hen each month; therefore each figure is the mean obtained for determinations on 8–15 eggs. The number in each group was regarded as insufficient for a separate statistical analysis; therefore the results for the entire period were analyzed statistically. It was shown that the effect of the level of PGA ingested on the amount found in the eggs of both chickens and turkeys was highly significant.

It is recognized that the techniques used for the liberation of PGA from the eggs may not have resulted in complete liberation of the vitamin. Some studies were conducted with the use of chick pancreas enzyme [3] or a hog kidney preparation (Bird et al., '46). Higher values were obtained with the use of these preparations in some cases; however the relative difference in the values for the eggs from the supplemented groups as compared to those from the low PGA group was

TABLE 2

Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of this vitamin found in the egg (1947 season).

LENGTH OF TIME ON EXPERIMENT	TURKEYS		CHICKENS	
	Basal ration	PGA supplemented ration	Basal ration	PGA supplemented ration
Months	*μg PGA/gm of whole egg*	*μg PGA/gm of whole egg*	*μg PGA/gm of whole egg*	*μg PGA/gm of whole egg*
1½	0.13	0.19	0.08	0.11
2½	0.09	0.15	0.09	0.16
3½	0.09	0.12	0.08	0.12
4½	0.16	0.30	0.15	0.17
5½	0.16	0.30	0.08	0.12
6½	0.06	0.18	0.11	0.13
7½			0.11	0.14
Total no. of eggs	44	43	59	59
Mean and S.E.	0.117 ± .007	0.209 ± .013	0.098 ± .004	0.133 ± .005
F Value	42.9 [1]		39.7 [2]	

[1] F Value required for significance at the 1% level is 6.93.
[2] F Value required for significance at the 1% level is 6.86.

consistent with the results obtained with takadiastase treatment. Therefore, in order to obtain comparable data during the experiment, the takadiastase technique was used throughout, and since equal numbers of eggs from each group of birds were included in each series of determinations, the relative values obtained are assumed to be valid. Variations in the amount found for any 1 month may be due in part to seasonal effects attributable to the level of egg production, feed con-

[3] Dr. L. R. Richardson generously supplied this preparation.

sumption, etc., but the figures may also represent variations in the amount of the vitamin liberated in the specific series of analyses carried out.

Blood studies. Analyses obtained on the "apparent" free PGA content of whole blood and plasma also demonstrated the influence of the dietary treatment. Results were obtained at the end of the experiments for both seasons on adult chickens and turkeys. In some cases, blood was taken from the same

TABLE 3

Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of "apparent" free pteroylglutamic acid found in the blood.

LENGTH OF TIME ON EXPERIMENT	SAMPLE USED	MILLIMICROGRAMS OF PGA/ML [1]		F VALUE	
		Basal ration	PGA supplemented ration	Observed	Required (1% level)
		Turkeys			
1946					
6 months	whole blood	9.0(12)	16.8(12)	17.8	7.88
	plasma	1.7(8)	6.0(8)	127.5	8.68
1947					
7 months	whole blood	8.4(16)	15.9(16)	19.2	7.53
	plasma	2.5(16)	7.4(16)	25.1	7.53
		Chickens			
1946					
2½ months	whole blood	10.2(14)	11.0(14)	not significant	
	plasma	3.2(13)	4.8(13)	13.0	7.77
1947					
7½ months	whole blood	9.7(8)	8.3(10)	not significant	
	plasma	3.8(8)	4.5(9)	not significant	

[1] The number of analyses is given in parentheses.

birds 2 weeks prior to the end of the experiment. These results are presented in table 3. The amount of "apparent" free PGA in the whole blood of the turkeys, for example, is significantly lower for the group fed the basal ration (8.6 mμg/ml) than for the group fed the supplemented ration (16.2 mμg/ml). In some cases the amount of free PGA in the cells and plasma for the 2 groups was determined. An average of 27.8 and 41.4 mμg of PGA per milliliter of cells from the defici-

ent and supplemented groups, respectively was found; 1.0 and 6.0 mμg of PGA per milliliter of plasma, respectively.

No difference was noted in the values for whole blood of the chickens between the 2 groups. The values for plasma reflected the influence of the dietary treatment in tests conducted during 1946, but no significant difference was noted in those conducted in 1947. More data are needed to explain this apparent discrepancy; however uncontrolled variables such as egg production, feed consumption and time of the year may have exerted an effect. Nevertheless, the data do demonstrate that the level of the microbiologically available vitamin in the blood of the chicken is relatively insensitive to the amounts of PGA fed, as compared to that available in the turkey. The values for the supplemented groups obtained in the present study are in excellent agreement with those obtained in previous work on the "apparent" free folic acid content of blood from various species (Schweigert and Pearson, '47). The higher values observed for the amount of the free vitamin in the blood of the avian species as compared to those obtained for others may be correlated with the higher requirement of the avian species for this vitamin.

Analyses obtained after enzymatic digestion were similar for whole blood and plasma for the 2 experimental groups. The following values were obtained for the amount of PGA in whole blood after enzymatic treatment for the basal and PGA supplemented turkeys: 56.1 mμg/ml (average of 16 determinations) and 64.7 mμg/ml (average of 16 determinations), respectively. For plasma, 57.9 and 57.8 mμg/ml were observed, respectively, for the 2 groups (14 analyses in each case). The amounts observed in chicken blood were as follows: 53.8 and 55.5 mμg/ml of whole blood for the basal and supplemented groups (average of 20 determinations in each case) and 38.9 and 36.1 mμg/ml of plasma, respectively (average of 14 analyses in each case). These rather surprising observations may be of great importance. It may be possible that animals that are very deficient in this vitamin may have sufficient amounts of the conjugated form (presumably that incre-

ment measured after enzymatic digestion) present in the blood and other tissues and are unable to break it down to the biologically active or free form. Work along this line will be conducted to determine the significance of this preliminary observation. From the data obtained on whole blood and plasma, it is apparent that most of the free vitamin occurs in the cells, while the amount of PGA observed after enzymatic digestion appears to be the same for both plasma and cells.

The greater differences noted in the PGA content of the eggs and blood for the 2 groups of turkeys as compared to the 2 groups of chickens suggest that the requirement of this vitamin is greater for the turkey than for the chicken.

Studies with the young poults. Preliminary experiments conducted in 1946 indicated that poults from hens receiving the low PGA diet grew at a slower rate than the poults from the adequately supplemented hens when the poults were fed rations similar to those fed the hens. In another experiment, a low PGA diet, high PGA diet and the standard flock ration were fed to 10 poults in each group. These poults were from hens fed an adequate ration. At about 4 weeks of age all birds in the low PGA group became infected with fowl pox, while those in the other 2 groups (located above and below the basal group in the brooders) did not become affected. Since this disease is considered very contagious, it seemed rather surprising that only the low PGA group was affected unless the type of ration fed influences the susceptibility. Much more work with carefully controlled conditions will be needed to verify this observation.

Poults hatched from eggs obtained after the breeding turkeys had been on experiment for 4, 5 and 6 months during 1947 were used to determine the effect of the dietary intake of the hens on the performance of the young poult (table 4). Purified rations were used in which the PGA content was varied from 0.2–2.0 mg/kg for the various groups (table 1). In the first experiment poults from hens that received either the basal ration or supplemented ration were used. All 9 poults from hens receiving the former ration died within 2 weeks when

fed 0.2 mg of PGA/kg of ration. However, all poults fed 2.0 mg/kg, regardless of the diet fed the hens, lived and grew at a normal rate. Three of 5 poults from hens fed the supplemented diet died when 0.2 mg of PGA/kg was fed and the remaining 2 poults grew normally. These observations were extended in 2 subsequent experiments in which groups were fed 0.8 mg of PGA/kg of ration in addition to groups fed 0.2 or 2.0 mg/kg. These results are summarized in table 4. It

TABLE 4

Effect of the amount of pteroylglutamic acid in the diet of the turkey hen and poult on the performance of the young poult.

EXP. NO.	PGA ADDED TO DIET		NO. OF POULTS	WEIGHT OF POULTS				NO. OF DEATHS[1]	NO. WITH PEROSIS
	hens	poults		1 day	1 wk.	2 wks.	3 wks.		
	mg/kg	*mg/kg*		*gm*	*gm*	*gm*	*gm*		
2	none	0.2	18	59	77	124	194	8	4
	2.0	0.2	7	57	78	145	234	none	2
	none	0.8	15	56	72	125	222	3	6
	2.0	0.8	6	58	89	173	285	none	2
	none	2.0	16	59	74	125	213	1	7
	2.0	2.0	7	61	81	146	250	1	1
3	none	0.2	6	56	85	125	199	2	1
	2.0	0.2	5	53	89	159	292	none	3
	none	0.8	6	54	89	225	332	none	2
	2.0	0.8	5	52	89	213	311	none	none
	none	2.0	5	59	84	149	298	none	none
	2.0	2.0	4	54	84	165	327	none	none

[1] Only those poults that died after 4 or more days on experiment are included in this tabulation.

will be noted that the mortality and also the rate of growth of the young poults were influenced by the diet of the hens from which the poults were obtained. From these experiments it can be seen that a total of 10 out of 24 poults died prior to 3 weeks of age when both the hens and poults received the diets low in PGA. However, either when the hens received the PGA supplemented diet or the poults received 2.0 mg of PGA/kg of ration, few losses occurred (2 poults out of 55). The mortality observed when 0.8 mg of PGA/kg of ration was fed to the poults was intermediate when the hens had been

fed the basal ration (3 out of 19). As indicated above, an excellent performance was noted when 0.8 mg of PGA/kg was fed and the hens had been fed the supplemented ration. The rates of growth observed also reflected the dietary treatment of the hens as well as that of the poults. The requirement of the poults appeared to be between 0.8 and 2.0 mg/kg of PGA judged on the basis of rates of growth and mortality when the hens had been fed diets low in this vitamin. It appears that 0.8 mg of PGA/kg is adequate for the poults when the hens had been fed a diet to which 2.0 mg of PGA had been added. Thus, these results may form a basis for explaining the differences in the requirements for PGA reported by Jukes and associates ('47) and Russell and Taylor ('47). The present work suggests that differences in the PGA nutrition of the hens may be a contributing factor in determining the amount required by the young poult.

Three of the poults from the hens fed the low PGA diet developed typical cervical paralysis (Richardson et al., '45; Jukes et al., '47) at 17–19 days of age when receiving 0.2 mg of PGA/kg. No other groups demonstrated this syndrome. The incidence of perosis was also higher for the poults from hens receiving the diet low in PGA, particularly in the second experiment. Thus, it has been demonstrated that feeding diets low in PGA to the hen adversely affects the ability of the young poult to survive and grow, and also increases the amount of the vitamin needed in the diet of the poult for normal development. These observations suggest that some of the mortality observed in young poults is due to subminimal levels of various nutrients, including PGA, present in the diet of the hens. Perhaps more attention should be focused on the development of improved rations for the breeding flock as well as for the growing birds.

Although no effect could be demonstrated on the egg production or hatchability due to ingestion by the hens of a diet low in PGA, the use of other criteria, namely, the levels of PGA in the eggs and blood and the performance of young poults from these hens suggests that the level of PGA fed

(0.42/kg of ration) was inadequate for the breeding turkey. The use of several criteria, therefore, in evaluating the nutritive value of the diet is highly desirable. With improvements in methods for liberating this vitamin from natural materials, more exact information on its distribution in feeds and stability will be forthcoming. Such information will facilitate more definite evaluation of the adequacy of a particular ration in terms of pteroylglutamic acid.

SUMMARY

1. The effect of feeding diets low and high in pteroylglutamic acid on the performance of adult chickens and turkeys and turkey poults was determined.

2. When a basal diet containing 0.42 mg of PGA/kg of ration was fed to turkey or chicken hens no detrimental effect on egg production, hatchability, hemoglobin level or general appearance was demonstrated as compared to when 2.0 mg of PGA were added/kg of ration.

3. The amount of PGA found in the eggs of both chickens and turkeys was markedly lower when the basal diet was fed as compared to the amount found when the diet supplemented with PGA was fed.

4. The "apparent" free folic acid content of the blood was also lower for the turkeys fed the low PGA diet while the level in the blood of the chickens was relatively insensitive to differences in the dietary treatment. The amount of PGA observed after enzymatic digestion of the blood samples, however, was shown to be approximately the same for both groups of chickens and turkeys, regardless of dietary treatment.

5. A higher mortality and slower rate of growth were noted when both young poults and the hens were fed diets low in pteroylglutamic acid than when a supplemented diet was fed either to the hens, to the poults, or to both.

6. For poults from hens adequately supplemented with PGA, 0.8 mg of the vitamin/kg appeared to be adequate; however, for poults from hens fed the diet not supplemented

with PGA, the requirement is apparently greater than 0.8/kg of ration.

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THE RELATION OF FED AND INJECTED TOCOPHEROLS TO DEVELOPMENT OF RANCIDITY IN THE STORED MEAT AND UTILIZATION OF CAROTENE BY THE RABBIT[1]

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(Received for publication August 18, 1947)

Work has been done to show that alpha-tocopherol, which prolongs the induction period of the fat, is stored in the tissues of the rat and turkey (Criddle and Morgan, '47; Hanson et al., '44; Lundberg et al., '44; and Mason, '42). Watts, Cunha and Major ('46) found that the amount of tocopherol stored in hog fat was too small a quantity to be of practical value, especially when the animals were fed a natural ration.

In the work reported here, a study was made of the relation of the tocopherols to the prevention of development of rancidity in the stored meat and fat of the rabbit. In addition, the effect of tocopherols upon the utilization of carotene was investigated.

A number of authors (Davies and Moore, '41; Hickman et al., '42, '44a, '44b; Moore, '40; and Jensen, '46) have reported a sparing action of tocopherol upon vitamin A. Guggenheim ('44), Hove ('43, '44), Jensen et al. ('43), Quackenbush et al.

[1] Published as Scientific Paper no. 733, Agricultural Experiment Stations, State College of Washington, Pullman, Washington.

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('41, '42), and Sherman ('42) have shown also that the tocopherols are effective in enhancing the utilization of carotene. Harris et al. ('44) found that 0.5 mg of natural mixed tocopherols was the optimum daily dose to demonstrate the sparing action of carotene on rats.

METHODS

Selection and care of animals

American white rabbits weighing 500–700 gm were used for this experiment. They were weaned when 4 to 6 weeks old and fed commercial rabbit pellets until all were eating, as evidenced by maintenance of weight. They were then separated according to sex and put into 3 comparable groups, and placed on the experimental diet. Supplements to the diet were withheld until the vitamin A stores were depleted, as evidenced by leveling off of the growth curve.

Diet and supplements

The composition of the purified diet is shown in table 1. Contrary to the findings of Mackenzie, Mackenzie and McCollum ('41), preliminary work with rabbits in our laboratory showed that fat was essential in the diet, both for utilization of carotene and general health and growth. For this reason lard was included in the diet.

Sufficient diet for 1 week was prepared at one time by mixing thoroughly all of the ingredients of the diet and then moistening the mixture sufficiently to form a pellet. The slightly moist pellets were spread on screens at room temperature overnight to dry and stored in closed tin cans for the week. The peroxide values of the diet at the beginning and end of the week were followed until it was apparent that there was no appreciable increase during this period. At first the yeast was ether-extracted, but this practice was soon discontinued because of the extremely low tocopherol content (Veron, '46). The diet was fed *ad libitum* in metal containers.

Supplements containing carotene, viosterol, and mixed tocopherols were prepared for each group weekly. Crystalline carotene (90% beta and 10% alpha) was dissolved in chloroform in 1-gram lots; the solution was taken up in ethyl laurate; and the chloroform was then evaporated on a water bath. The volume of the carotene solution was adjusted with ethyl laurate so that 1 ml of the solution contained 20 mg carotene. It was then stored in brown bottles in the refrigerator. Aliquots of this solution were used in mixing the weekly supplement. The vitamin D used was viosterol in oil with

TABLE 1

Composition of rabbit diet.

BASAL DIET		SUPPLEMENTS		
			Tocopherol	
			low	high
	%		*mg/day*	*mg/day*
Casein	15	Tocopherol	1.0	11.1
Brewers' Yeast	15	Viosterol	1.0	1.0
Lard	10	Carotene	3.0	3.0
Wheat Bran	15			
Salt Mix[1]	6			
Cornstarch	39			

[1] Salt mix no. 51 (Mackenzie, Mackenzie and McCollum, '39).

standardized potency of 10,000 U.S.P. units per gm. A tocopherol concentrate containing 34% mixed tocopherols was used.

For the groups fed high levels both the viosterol and tocopherol were weighed directly into brown bottles, the carotene solution added, and the volume adjusted with ethyl laurate so that 0.3 ml was fed at each supplementation. The mixed supplement was then stored in the refrigerator. This supplement was fed by pipette directly into the animal's mouth 3 times weekly.

For the groups of animals receiving injected tocopherol, the viosterol and carotene were prepared and fed according to the method followed for the previous groups. The tocopherol, however, for this group was weighed out and the volume adjusted with ethyl laurate so that 0.5 ml contained the weekly

supplement which was injected with hypodermic syringe into the heaviest portion of the right thigh once weekly. All injections were kept within a 1-inch circle on the thigh.

The daily tocopherol requirement of the rabbit has been found to be between 0.2 mg and 1.0 mg per kilogram body weight (Eppstein and Morgulis, '40, '42; Friedman and Mattill, '41; Hove and Harris, '47; Mackenzie, Levine and McCollum, '40; Mackenzie and McCollum, '39, '40). The amount of 1.0 mg mixed tocopherol was chosen for the low level group to avoid any incidence of dystrophy.

Treatment of the meat

At the end of the experimental period, the rabbits were dressed, the livers and free fat removed and frozen, and the meat cut from the bones and frozen. The right thigh of the animals which had had the tocopherol injected was removed and stored separately. After freezing, the meat of each animal was ground and the sausage mixed thoroughly. It was found much easier to get a fine grind, free from stringy tissue, if it was ground while still frozen. The sausage was weighed out into 50 gm lots, which were made into balls of similar size and shape and placed in freezer cartons with strips of wax paper between them to facilitate removal in individual balls for analysis. The cartons were then put into frozen storage at —18°C.

Rancidity tests on the frozen sausage

The samples were analyzed at intervals until the sausage from each animal was found rancid. The fat was extracted from the sausage by blending 50 gm sausage in a Waring blendor for 1 minute with 100 mg chloroform and 70 gm of anhydrous sodium sulfate. The extract was filtered through Whatman no. 2 filter paper, and a 5 ml sample of the clear filtrate dried to determine fat content. The amount of the filtrate necessary to make 0.5 gm fat was used for the test. A peroxide value above 10 was designated rancid. The

method used for determination of peroxide values throughout the experiment was reported by Watts and Major ('46).

Vitamin A and carotene analyses

Vitamin A analyses were made on all livers using the Carr-Price reaction as reported by Davies ('33). This was changed to the Gallup and Hoefer method ('46) when good agreement was found between the two. Carotene was determined by the Wall and Kelley method ('43).

Determination of pH

Five grams of sausage were mixed thoroughly with 50 ml distilled water. After 30 minutes the pH was read on a Beckman meter.

EXPERIMENTAL RESULTS AND DISCUSSION

This work was carried out in 2 separate experiments. Experiment 1 included 3 groups of animals fed the purified diet. Group 1 received a low-level tocopherol supplement; group 2, a high-level tocopherol supplement; and group 3, a high-level tocopherol supplement by injection. Experiment 2 was concerned with animals fed a natural diet which was supplemented with injected tocopherol and tocopherol phosphate.

Experiment 1

Part A. This experiment was set up using the purified diet shown in table 1. In addition to the effect of fed and injected tocopherol on development of rancidity, the effect of fed tocopherol on the utilization of carotene was estimated.

The effect of tocopherol upon rancidity development in the stored meat is summarized in table 2. At the end of 12 months' storage, all but one of the samples from the group fed low level tocopherol were rancid (mean peroxide value of 14), whereas none of those from the group fed high level tocopherol were rancid (mean peroxide value of 4.4). The mean peroxide value of the group which had the tocopherol injected was

between the average of these 2 groups, or 5.6. A statistical analysis of these figures shows that the difference between the average peroxide values of the high level of tocopherol, whether fed or injected, and the low level of tocopherol is significant at the 1% level.

TABLE 2

Effect of feeding and injecting tocopherol on the rancidity development of the meat.

CODE NUMBER	TOCOPHEROL	PEROXIDE VALUES AFTER STORAGE FOR		pH OF MEAT
		12 mo.	18 mo.	
	mg/day			
32	1.0	18.0		5.50
39	1.0	8.0	13.5	5.70
45	1.0	16.0		5.70
47	1.0	14.0		5.69
		Mean 14.0		
33	11.1	7.2	14.0	6.15
34	11.1	4.0	9.0	5.55
37	11.1	3.4	5.0	5.90
40	11.1	3.0	5.0	5.50
	Injected	Mean 4.4[1]	8.2	
35	11.1	3.0	6.5	6.00
36	11.1	7.8	9.0	6.04
38	11.1	4.0	6.5	5.85
42	11.1	5.0	5.5	5.70
43	11.1	7.0	8.5	5.70
44	11.1	7.0	9.0	5.62
		Mean 5.6[1]	7.5	

[1] Significant at the 1% level. The method for determining significance was taken from Snedecor, G. W., *Statistical Methods.*

It has been reported that tocopherol in the diet is necessary to prevent the destruction of vitamin A and carotene in the digestive tract. More vitamin A was stored in the liver as well as more carotene excreted in the feces when the dietary tocopherol was increased (Guggenheim, '44; Harris et al., '44; Hickman et al., '42; Hove, '43). A carotene balance was run to see if the same conditions were necessary for carotene utilization for rabbits as had been reported for rats.

Before the carotene balance was started, the animals had been maintained on the same supplements for a period of 30

days or more. A 4-day period was used as a test period, and feces were collected at a stated hour daily. Feces that could not be analyzed for carotene content immediately after collection were frozen until an analysis could be made.

The results of the vitamin A storage and carotene excretion from this experiment are shown in table 3. The lower figure of carotene excreted by the group of animals fed a low level of tocopherol appeared to be due to an extremely small amount of feces on the first day of collection (4 gm as compared to a daily average of 35 gm), rather than to any difference in

TABLE 3

Effect of tocopherol on excretion of carotene and storage of vitamin A.

PART	GROUP	NO. OF ANIMALS	SUPPLEMENTS FED		CAROTENE EXCRETED (AVERAGE)	AVERAGE VITAMIN A STORED IN LIVER	
			Tocopherol	Carotene			
			mg/day	*mg/day*	*mg/animal/day*	*mg/gm*	*total mg*
A	low	4	1.0	3.0	0.943	0.020	2.116
	high	4	11.1	3.0	1.597	0.018	1.570
	injected	6	11.1	3.0	1.327	0.014	1.298
B	low	6	1.0	1.5	0.688	0.012	1.231
	high	7	11.1	1.5	0.414	0.010	1.234

carotene utilization. The carotene content of the feces from this group on the day mentioned was even lower per gram than that on other days.

There was no significant difference between the 3 groups in either carotene excretion or vitamin A storage in the liver.

Part B. Since the results of the carotene balance and of the vitamin A assay on the livers (shown in table 3, part A) did not agree with those reported by Guggenheim on rats ('44), it was thought that another set of animals should be used to investigate the effect of the amount of tocopherol fed on carotene excretion, utilization, and rancidity development in meat. Because of the high quantity of carotene excreted and vitamin A stored in the liver, it was believed that the amount fed in part A was too far in excess of the requirement

to be a good measure of the effect of tocopherol (Moore, '40). Therefore, for this trial, the carotene level was decreased from 3.0 mg daily to 1.5 mg daily. Because Fraps and Meinke ('45) and Callison and Orent-Keiles ('46) had reported that the availability of carotene was greater when fed in an oil, the supplements were mixed and fed in peanut oil [4] instead of in ethyl laurate.

The results on carotene excretion and vitamin A storage for both parts of this experiment are shown in table 3. In neither experiment did the greater amount of tocopherol fed result in significant change in amount of carotene excreted nor in the amount of vitamin A stored in the liver. The tocopherol supplement, even in the low level, may have been sufficient to exert protective action on the carotene. More work is needed before conclusions can be drawn on the effect of feeding the supplements in oil instead of in ethyl laurate. However, some fat, either in the diet or in the supplements, appeared necessary in order to get any storage of vitamin A in the liver. In preliminary work where both were omitted, no vitamin A was found in any of the livers, although 3.0 mg carotene was fed daily. This was found to be in agreement with other work (Callison and Orent-Keiles, '46; Hove and Harris, '46; Quackenbush et al., '41, '42).

An accelerated method for development of rancidity in the ground meat was devised and compared with frozen storage results. This method consisted of mixing thoroughly 4.0 ml of chloroform with 25.0 gm of the ground meat ready for freezing. The mixture was put into 250 ml beakers in a layer of uniform thickness, the beaker capped with aluminum foil to prevent vaporization of the chloroform, and stored in an incubator at 30°C. In this way these samples could be stored for periods as long as 60 hours without apparent bacterial spoilage. Peroxide values were run on samples stored in this way at 36- and 48-hour intervals.

The results of the accelerated tests on the meat are shown in table 4. At the end of the 36-hour period all but one of

[4] Obtained through the courtesy of Planters Edible Oil Company, Suffolk, Va.

the meat samples from animals fed the low tocopherol level were rancid and that one was near the point of rancidity (average 17.1). On the other hand, only one of the samples from the animals fed high tocopherol showed any development of rancidity at the end of the 36-hour period (average 2.8). Statistical analysis shows that this difference is significant at the 1% level. The early development of rancidity in the one

TABLE 4

Comparison of accelerated and frozen storage tests on sausage.

CODE NUMBER	TOCOPHEROL FED	PEROXIDE VALUES			pH OF MEAT
		Accelerated		Frozen storage	
	mg/day	*36 hr.*	*48 hr.*	*6 mo.*	
63	1.0	13.7	19.5	11.5	5.83
66	1.0	23.2	37.0	11.5	5.80
69	1.0	26.5	41.5	21.0	5.72
70	1.0	20.0	35.0	14.5	5.60
72	1.0	8.5	22.0	8.5	5.65
74	1.0	10.5	23.5	9.5	5.73
		Mean 17.1 [1]	29.6	12.7	
61	11.1	12.5	25.0	11.0	5.73
62	11.1	0.1	1.5	1.0	5.70
64	11.1	3.5	4.3	0.1	5.72
65	11.1	0.5	0.7	3.5	5.83
68	11.1	0.0	0.5	3.5	5.70
71	11.1	3.0	4.0	4.5	5.70
73	11.1	0.0	3.7	5.0	5.68
		Mean 2.8 [1]	5.7	4.1	

[1] Significant at the 1% level.

sample cannot be explained on the basis of pH difference (Watts and Peng, '47a). A colorimetric comparison of filtrates of the meat (prepared by blending 100 gm sausage with 140 ml water) from the different animals showed no higher hemoglobin content in this sample (Watts and Peng, '47b).

Peroxide values were determined on the meat after 6 months in frozen storage. The relative values, as shown in table 4, agreed with those made earlier by accelerated tests.

Experiment 2

This experiment was set up to determine the effect of injected tocopherol and tocopherol phosphate on rancidity development in the stored meat of rabbits fed a natural diet.

The diet used was a commercial rabbit chow [5] with the following guaranteed percentage composition: crude protein, not less than 14.5%; crude fat, not less than 2.0%; crude fiber, not more than 18.0%; nitrogen free extract, not less than 44% and ash, not more than 7.5%. The ingredients were ground oats, corn meal, soybean oil meal, corn germ meal, alfalfa meal, wheat gray middlings, molasses, riboflavin supplement, 1.5% calcium carbonate, and 0.5% iodized salt.

The animals selected for this experiment were 3 to 4 months old and had nearly reached full growth. They were divided into 2 lots; each lot consisted of a control group receiving no supplements and an experimental group. Lot 1 experimental group was injected with 200 mg of mixed tocopherol in ethyl laurate at the beginning and mid-point of a 3-week period. For lot 2 experimental group, 200 mg of water-soluble tocopherol phosphate was injected at these same intervals.

At the end of the 3-week period the animals were dressed and the right thigh of the injected group separated before storage, as has been described under the method.

The results of the experiment are shown in table 5. In the frozen storage tests of the ground meat no difference was shown between the animals injected with tocopherol or tocopherol phosphate and those of the control groups receiving no supplementary tocopherol. However, when compared to the rest of the tissue, protection from rancidity development was shown, in both lots, in the thigh muscle at the site of injection of the tocopherol.

It may be that the time between the injection of the tocopherol and killing of the animals was too short to allow for storage throughout the tissues, although Eppstein and Morgulis ('42) found that a 3–10-day period was sufficient

[5] Distributed by the Ralston Purina Company.

to cure rabbits of advanced dystrophy when they administered the water soluble disodium-α-tocopherol phosphate by injection. This experiment allowed fully the 10-day period suggested by Eppstein and Morgulis. Hove and Harris ('47) also found that injected tocopherol-phosphate brought about the cure of muscular dystrophy in the rabbit in a 5–25-day period.

TABLE 5

Effect of injecting tocopherol and tocopherol-phosphate. (Mature animals on natural diet.)

CODE NUMBER	AMT. TOCOPHEROL INJECTED (MG) Mixed tocopherols	STORED MEAT PEROXIDE VALUES			pH OF MEAT
Lot 1		*5 mo.*	*7 mo.*	*9 mo.*	
21	0.0		16.0		6.60
22	0.0		12.0		6.20
23	0.0		8.0		6.17
24	400.0		7.0		6.10
25	400.0		17.0		6.11
25 [1]				11.0	
26	400.0		5.0		6.03
Lot 2	Tocopherol phosphate				
50	0.0	2.8			6.14
51	0.0	2.8			5.90
52	0.0	15.7			5.91
53	400.0	72.7			5.96
54	400.0	20.0			5.80
54 [1]				7.5	
55	400.0	29.5			5.78
55 [1]				9.5	
56	400.0	7.2			6.02
56 [1]				4.5	

[1] Muscle at site of injection.

SUMMARY AND CONCLUSIONS

The relation of tocopherols in the diet to development of rancidity in the fat and meat and to the utilization of carotene was investigated.

1. When high levels of tocopherol were fed to or injected in rabbits on a purified diet, protection from the development of rancidity was shown.

2. On a natural ration, no protection from the development of rancidity was shown when the animals were injected with tocopherol or tocopherol phosphate 3 weeks before slaughter.

3. Accelerated tests for the development of rancidity on sausage preserved with chloroform agreed with tests made on sausage held in frozen storage.

4. No difference either in the utilization of carotene or in the liver storage of vitamin A was observed on supplementing a synthetic diet with high and low levels of tocopherol.

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THE EFFECT OF DIETARY RESTRICTION OF B-COMPLEX VITAMINS AND PROTEIN ON THE EXCRETION OF CREATININE BY HUMAN SUBJECTS [1, 2]

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FOUR FIGURES

(Received for publication October 10, 1947)

The work of Folin ('05), Hoogenhuyze and Verploegh ('05), Klercker ('07), and Shaffer ('08) some 40 years ago led to the formulation of several concepts which, in spite of intensive investigation and much individual disagreements, still are of interest to the nutritionist: (1) that some biochemical phenomena proceed at similar, apparently constant,

[1] This investigation was carried out under a contract between the Office of the Surgeon General, U. S. Army, and Passavant Memorial Hospital, Chicago, Ill.; it was also supported in part by grants from the Clara A. Abbott Fund of Northwestern University Medical School, and from the Nutrition Foundation, Inc.

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rates in all healthy physically fit individuals, quite independent of the usual variations of the normal diet and physical activities and, therefore, may be used as "yard sticks" for assessing the total active mass of certain types of tissues; and (2) that alterations of structure or functional efficiency of such tissues resulting from disease, malnutrition or other debilitating conditions may be reflected in altered rates of the basic reactions.

Although the first is opposed to modern concepts of extreme complexity and variability of biochemical phenomena, nevertheless, the early investigators demonstrated such an apparent constancy in the case of creatinine in relation to the unit weight — the creatinine coefficient — which presumably is related to the muscular mass (Shaffer, '08; Hodgson and Lewis, '26; McClugage, Booth and Evans, '31). This was all the more surprising since the conclusions were based upon determinations of the daily rate of urinary excretion which, in addition to cellular mechanisms, involves renal function. In recent years the role of protein and amino acids in the synthesis of creatine and creatinine has received particular attention. The result has been an increasing emphasis on the variation rather than on the uniformity of the creatinine excretion. The changing point of view is evident in the reviews of Hunter ('27), Peters and Van Slyke ('46) and Beard ('41).

The differences in the average daily rate of excretion were explained by Folin and Shaffer largely on the basis of the second postulate. This was perhaps best stated by Shaffer ('08) that "in a muscle in a high state of nutrition and development, certain processes which cannot be fully defined at present, but which lead to the formation of creatinine as a waste product, are proceeding at a greater speed than in a muscle organically weak or diseased" and that upon the intensity (see Hoogenhuyze and Verploegh, '05) of these processes, as measured by the creatinine coefficient, "appears to depend the muscular efficiency of the individual." Because of the experimental difficulties and the long time required in

such investigations, these implied relations have not been studied in a satisfactory manner in human subjects.

In the course of an experiment with 7 initially physically fit young men (Berryman et al., '47), an opportunity was presented for applying uniform procedures to a study of the variations of creatinine excretion and their relation to changes in weight and physical performance over a period of 50 weeks as follows: a "normal" period of 11 weeks; an experimental period of 8 months (36 weeks) during 4 of which the diet was restricted with respect to protein and B-complex vitamins, followed by 4 months in which a program of supplementation was instituted in accordance with individual needs; and a final period of 3 weeks, during which all subjects received approximately equal quantities of a rehabilitation diet. Two of the subjects served as "controls" on the effect of the restricted basal diet plus all of the supplements during the 2 periods of 4 months each. Thus, the experiment provided data on the "constancy" of the creatinine coefficient under controlled conditions and the effect of subsequent changes in the "state of nutrition."

EXPERIMENTAL

Since the experimental conditions were described fully by Berryman et al. ('47) in connection with other data, only the salient features will be given here. The 7 men, ages 22 to 27 years, were chosen from many volunteers on the basis of physical and mental qualities. All subjects received equal weighed quantities of "normal" diet I during the first 11 weeks, during which a uniform program of training and test procedures was instituted and an apparent plateau of physical performance was attained. The diet contained (Berryman et al., '47): 3170 cal.; 70 gm protein (including protein from 75 gm lean beef, 225 ml milk and 1 egg); 1.44 mg thiamine; 1.84 mg riboflavin; 15.6 mg niacin; 44 μg biotin; 64 μg L. casei factor; 4.7 mg pantothenic acid; 1.7 mg pyridoxine; and adequate quantities of ascorbic acid, vitamin A, calcium, phosphorus, and iron.

The basal weighed experimental diet II was then given to all subjects: a total of only 5 weeks (12 to 16, inclusive) to subjects 3 and 4, and 36 weeks (12 to 47, inclusive) to the other 5 subjects. This permitted a determination of the initial creatinine excretion on a relatively creatine and creatinine-free diet by the 5 experimental subjects and *subjects 3 and 4*

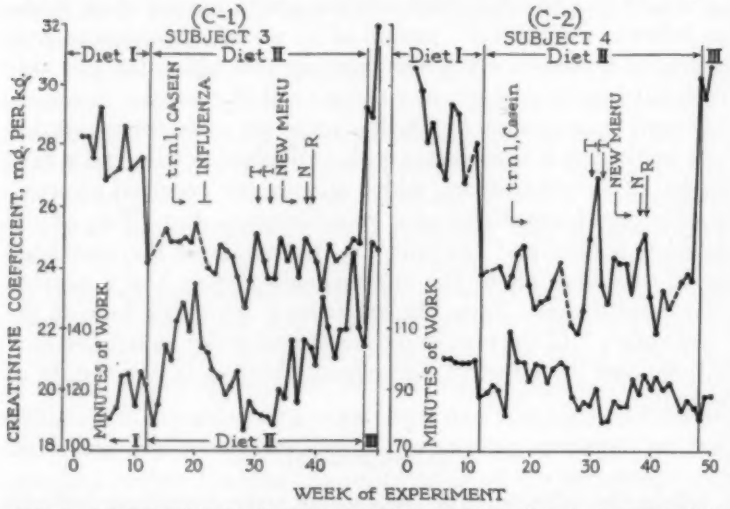

Fig. 1 Control subjects. Creatinine coefficient and work output expressed in total minutes of work to exhaustion in 2 work periods. The letters refer to vitamin supplementation — small letters to vitamins given orally in tablets 3 times per day in quantities sufficient to bring the total intake to the level of diet I; capital letters to vitamins given intravenously in large doses — as follows: t or T, thiamine; r or R, riboflavin; n or N nicotinamide; l, "lesser known" B-complex vitamins, pyridoxine, pantothenic acid, biotin, and L. casei factor.

who subsequently served as "controls" on the effect of the basal diet plus supplements. The diet contained: 3300 cal.; 45 gm protein (including animal protein only from salt pork without visible lean meat); 0.50 mg thiamine; 0.30 mg riboflavin; 5.8 mg niacin; 19 μg biotin; 23 μg L. casei factor; 1.1 mg pyridoxine; 1.1 mg pantothenic acid; and adequate quantities of ascorbic acid, vitamins A and D, and minerals either in the diet or in supplements.

Supplements were then given to the control subjects 3 and 4: 40 gm of protein in the form of calcium caseinate and the 7 crystalline vitamins indicated in both diets above, in quantities equal to that of diet I. These subjects later received

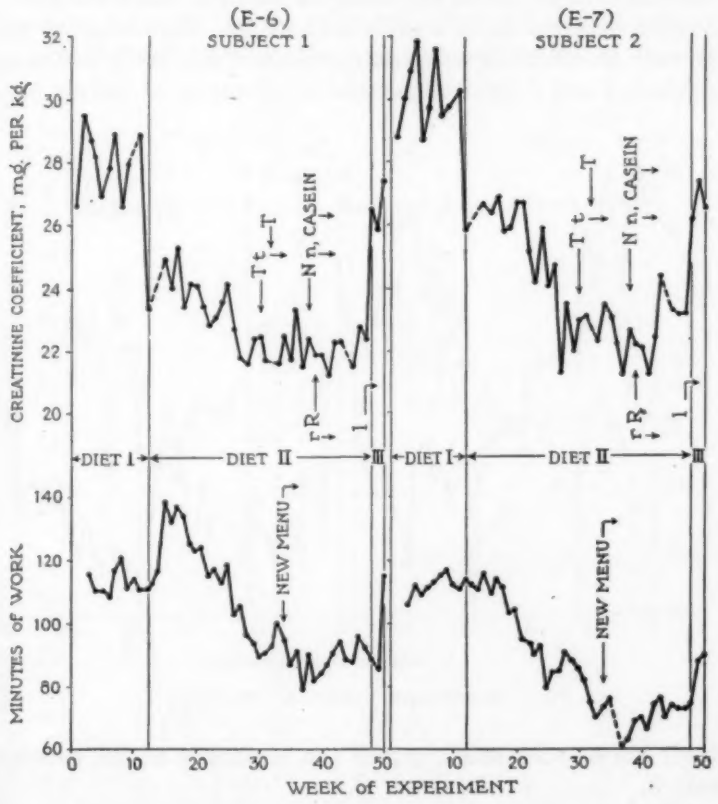

Fig. 2 Experimental subjects 1 and 2. See figure 1.

intravenous injections of vitamins at the same time as the experimental subjects (see capital letters in figures 1 to 4).

The month in which protein supplementation was instituted in the 5 experimental subjects is shown in tables 1 and 2; the

order of vitamin and other supplementation is indicated in figures 1 to 4. This had to be changed somewhat in individual cases because of the symptoms which developed in some subjects. Thus protein (casein) supplementation was instituted as follows: at the beginning of the fifth month to experimental subjects 5, 6, and 7, and at the beginning of the seventh month to experimental subjects 1 and 2. In addition, subjects 6 and 7 received further supplements of 100 gm lean

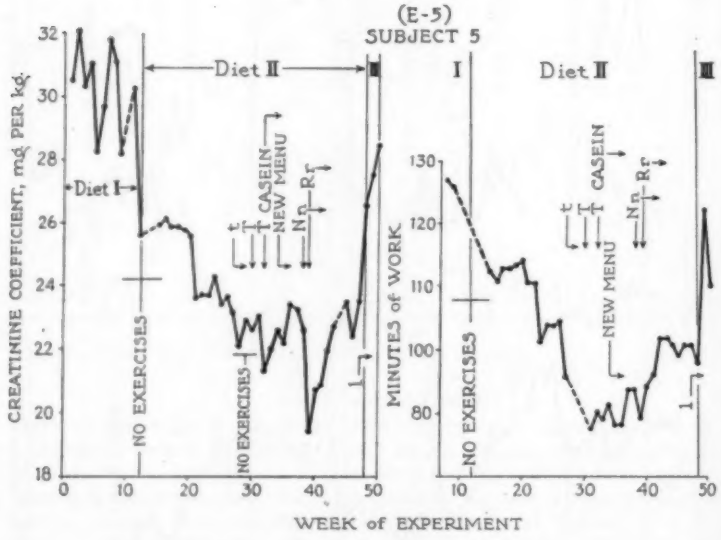

Fig. 3 Experimental subject 5. See figure 1.

beef, 225 ml milk and 1 egg at the beginning of the seventh month.

Rehabilitation diet III was given during weeks 48 to 50, inclusive. It contained 250 to 350 gm meat, 1 to 1.5 liters milk, 1 to 2 eggs, dried brewers' yeast, and, in addition, all of the crystalline B-complex vitamins. All subjects were served the same generous portions and, although they were allowed to ask for additional food, they seldom ate more. Thus, the in-

take of food, although not weighed as in the previous diets, was approximately the same for all subjects.

Weight. This was determined each morning after urine had been voided at 7 A.M. and before any water had been taken.

Work output. This was determined with a calibrated electro-dynamic brake bicycle (Kelso and Hellebrandt, '34). Work was performed twice to the point of exhaustion of the leg

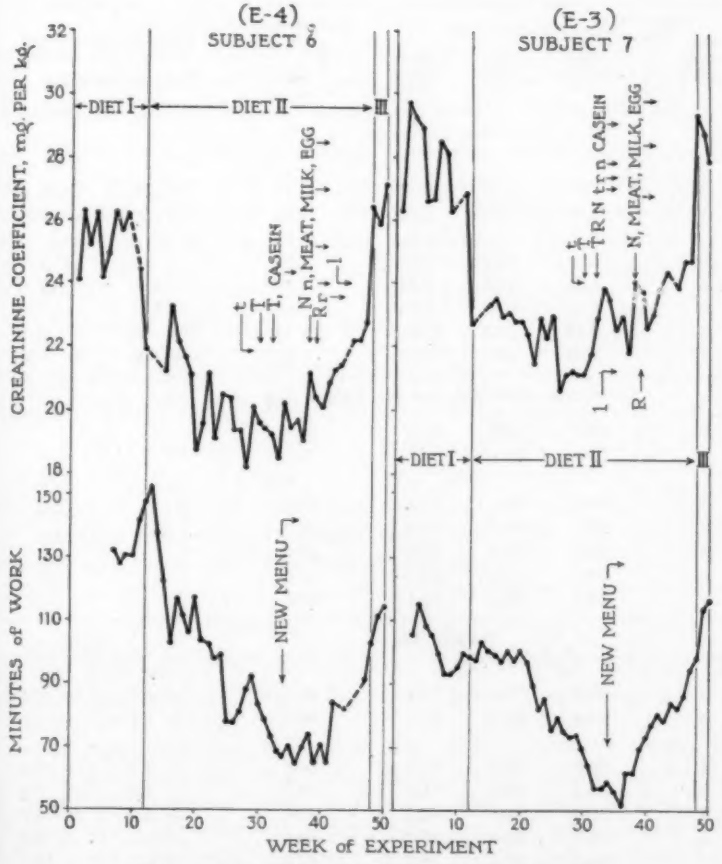

Fig. 4 Experimental subjects 6 and 7. See figure 1.

muscles, with a rest period of exactly 10 minutes between the tests (Foltz, Ivy and Barborka, '42). Previous experience had shown that the rate of pedaling is very important in obtaining comparative results from many subjects. Accordingly, throughout the year all subjects pedaled at a rate of 92 ± 4

TABLE 1

Effect of normal and rehabilitation diets. Monthly averages.

SUBJECT NO.	NORMAL DIET I			REHABIL. DIET III	DIFFERENCES BETWEEN RESULTS OF I AND III	
	July Weeks 1–2	Aug. Weeks 3–7	Sept. Weeks 8–11	June Weeks 48–50	Sept. and June	
	Creatinine, gm per day					
					mg	%
3	2.27	2.15	2.13	2.41	+ 280	+ 13.1
4	2.04	1.87	1.84	2.02	+ 180	+ 9.8
1	1.76	1.77	1.73	1.69	— 40	— 2.3
2	2.16	2.21	2.14	1.88	— 260	— 12.1
5	2.21	2.08	2.04	1.89	— 150	— 7.4
6	2.16	2.13	2.12	2.18	+ 60	+ 2.8
7	1.96	1.89	1.80	1.93	+ 130	+ 7.2
	Creatinine coefficient, mg per kg per day					
3	28.3	27.6	27.9	30.0		
4	30.3	28.1	28.0	30.2		
1	28.1	28.1	27.8	26.6		
2	29.4	30.5	29.8	26.7		
5	31.3	30.2	29.8	27.5		
6	25.2	25.4	25.5	26.5		
7	28.2	28.0	27.1	28.6		
	Weight, kg					
					kg	%
3	80.1	77.9	76.8	80.2	+ 3.4	+ 4.4
4	67.4	66.5	65.8	66.9	+ 1.1	+ 1.7
1	62.8	62.5	62.3	63.6	+ 1.3	+ 2.1
2	73.5	72.3	71.7	70.3	— 1.4	— 2.0
5	70.5	68.8	68.3	68.8	+ 0.5	+ 0.7
6	85.7	84.0	83.2	82.2	— 1.0	— 1.2
7	69.8	67.6	66.6	67.3	+ 0.7	+ 1.1

TABLE 2

Effect of restricted diet and supplements. Monthly averages.

The initiation of protein supplementation is indicated by *c* (45 gm casein) and *m* (100 gm lean beef, 225 ml milk, 1 egg). The numbers in parentheses in the next to last column indicate the month in which the minimum was attained; for example, (1) and (2) represent January and February, respectively.

SUBJECT NO.	OCT. Weeks 12–16	NOV. Weeks 17–20	DEC. Weeks 21–24	JAN. Weeks 25–29	FEB. Weeks 30–33	MARCH Weeks 34–37	APRIL Weeks 38–41	MAY Weeks 42–46	CHANGE Jan.	CHANGE Minimum	CHANGE May
	Creatinine, gm per day									% of initial (Oct.=100%)	
3	1.90	1.91*c*	1.83	1.81	1.82	1.85	1.86	1.91	— 4.7	— 4.1(1)	+ 0.5
4	1.56	1.57*c*	1.49	1.46	1.58	1.54	1.52	1.51	— 6.4	— 6.4(1)	— 3.8
1	1.51	1.51	1.44	1.40	1.35	1.38	1.37*c*	1.42	— 7.3	—10.6(2)	— 6.0
2	1.88	1.84	1.74	1.58	1.54	1.48	1.47*c*	1.59	—16.0	—21.8(4)	—15.4
5	1.78	1.75	1.59	1.51	1.42*c*	1.47	1.37	1.51	—15.2	—23.0(4)	—15.2
6	1.84	1.71	1.63	1.54	1.47*c*	1.50	1.59*m*	1.68	—16.3	—20.1(2)	— 8.7
7	1.54	1.50	1.44	1.39	1.41*c*	1.37	1.44*m*	1.56	— 9.7	—11.0(3)	+ 1.3
	Creatinine coefficient, mg per kg per day										
3	24.8	25.1*c*	24.2	23.8	24.2	24.5	24.3	24.7			
4	23.8	24.2*c*	23.1	22.7	24.6	24.0	23.7	23.4			
1	24.1	24.3	23.3	22.5	21.9	22.3	21.9*c*	22.2			
2	26.3	26.3	25.5	23.1	22.8	22.5	22.1*c*	23.3			
5	25.9	25.8	23.8	23.0	22.3*c*	22.9	20.9	22.7			
6	22.2	21.0	20.2	19.5	19.2*c*	19.7	20.6*m*	21.8			
7	23.2	22.9	22.2	21.4	22.4*c*	22.7	23.3*m*	24.2			
	Weight, kg										
3	77.0	76.3*c*	75.5	75.9	75.2	75.6	76.6	77.4	—1.4	—2.3(2)	+0.5
4	65.9	64.9*c*	64.8	64.5	64.4	64.1	64.3	64.6	—2.1	—2.7(3)	—2.0
1	62.9	62.3	62.0	62.1	61.3	61.1	62.6*c*	63.6	—1.3	—2.9(3)	+1.1
2	71.7	69.9	68.3	68.3	67.5	65.7	66.6*c*	68.3	—4.7	—8.4(3)	—4.7
5	68.8	67.8	66.8	65.7	63.6*c*	64.0	65.6	66.6	—4.5	—7.6(2)	—3.2
6	83.1	81.6	80.8	78.7	76.5*c*	76.1	77.0*m*	77.3	—5.3	—8.4(3)	—7.0
7	66.7	65.7	65.0	64.0	62.0*c*	60.5	61.6*m*	64.6	—4.0	—9.3(3)	—3.8

revolutions per minute. In order to eliminate the complication of increasing performance due to training, all subjects performed the test 3 times each week, along with other similar tests during the first 2 months. Thereafter, the activities were decreased and the work output was determined only once each week. During the first month, the field strength of the generator, or the resistance against which the work was performed, was so adjusted that each subject became exhausted within 50 to 60 seconds in each of the 2 work periods. The rate of work finally adopted was 19.1 kgm per second for subjects 1 and 5, and 23.8 kgm per second for subjects 2, 3, 4, 6, and 7.

Urine. Collections were made 4 days each week in exactly 24-hour periods, from 7 A.M. Tuesday to 7 A.M. Saturday. The 1-gallon amber glass jugs were kept in refrigerators at all times. The volume was measured each morning, and exactly one-tenth volume was placed into a bottle containing the 4-day composite sample. The latter was kept in the deep-freeze unit.

Creatinine. The photoelectric method of Peters ('42) was used. Dilutions were made to give galvanometer deflections in the range of 40 to 60 units, using the Evelyn colorimeter. A difference of 1.0 unit, from 59 to 60, represents an error of 3.2%; from 40 to 41, an error of 2.8%. In 100 consecutive duplicate analyses, the mean analytical error was well within $\pm$ 1%. The procedure was standardized at intervals and checked by means of recoveries of added creatinine, using creatinine-zinc chloride and creatinine picrate. Throughout the experiment, all determinations were made by the same analyst.

RESULTS

The most rapid changes in weight during the first period occurred in July and August while the subjects were undergoing training. However, the greatest losses were noted during the first week. This is not apparent in the average results given in table 1. Thereafter, the weight remained well within

± 1 kg during September and the succeeding 2 months of the restricted diet II (table 2).

The weight of 4 of the subjects (2, 5, 6, and 7) began to fall quite rapidly in December (the third month of the restricted diet) when early symptoms of deficiency began to appear. Diet II became monotonous, and at this time all of the subjects began to refuse some of the corn meal biscuits and the canned vegetables. The quantity of food thus left unconsumed was variable from day to day. It was small at first, but became larger during January, amounting to from 50 to as high as 800 cal. on some days, with an average of about 200 cal. The control subjects, particularly subject 3, refused as much food as the experimental subjects. They ate all of the food when a new attractive menu, which had the same ingredients as before, was given after March 1.

The work output, expressed in minutes required for exhaustion in 2 work periods, is shown in figures 1 to 4. The test is not entirely objective; it is subject to training, and periods of "staleness" and, like all physical efficiency tests, requires cooperation. However, it is improbable that any of these factors was of major importance because of the precautions taken against the occurrence of staleness and continued training response, and because of each subject's conscientious personal desire to do his best in each test. The parallel changes which were observed with other tests during the periods of restriction and supplementation were discussed in a previous paper (Berryman et al., '47).

Excretion of creatinine and the creatinine coefficient

Weekly variations. Since the variations of weight were small from week to week, the coefficient generally paralleled the total daily excretion. The results shown in figures 1 to 4 represent the average creatinine coefficients over periods of 4 days each week. Although the average tended to minimize the daily variations, considerable change was noted from week to week. The results are plotted on a large scale, which thus exaggerates the differences, and perhaps can be judged

best by the calculated coefficients from control subjects 3 and 4 during the first 2 months of diet II. Thus, the maximum deviations from the mean of all determinations during October and November were: — 3.0 and + 2.2% from subject 3; — 2.5 and + 3.3% from subject 4. In the case of subject 3, the maximum deviations remained within this range during the entire 8 months of diet II; however, they became greater in later months in the case of subject 4, the greatest differences of — 6.2 and + 11.1% of a 2-months' average occurring during February and March.

The maximum variations of the coefficients from the 2-months' mean of each subject were somewhat greater with diet I which contained creatinine. Thus, during August and September, the maximum percentage deviations, in the order of subjects given in the tables, were: — 3.1, + 5.6; — 4.5, + 4.8; — 4.8, + 3.2; — 4.8, + 3.4; — 6.0, + 3.7; — 4.9, + 3.3; — 4.9, + 6.0.

Thus, the tendency toward variations was greater in some subjects than in others, which is evident not only in our data (figs. 1 to 4) but also in those of Folin ('05), Hoogenhuyze and Verploegh ('05), and Shaffer ('08).

In many instances, the excretion increased or declined in a regular manner over periods of several weeks during the administration of diet I. These phenomena did not occur simultaneously in other subjects. Other instances of regular decline or rise were noted during the long period over which the restricted diet II was administered.

Monthly variations. Normal diet I and first month of diet II; 8 months of diet II in case of subjects 3 and 4. Because of the weekly variations in creatinine excretion, which often appeared to be rhythmic, a certain degree of variation in the monthly averages was to be expected. The total excretion generally decreased during the 3 months of diet I (table 1).

All subjects excreted considerably less creatinine after initiation of the almost creatinine-free diet II. Thus, they excreted an average of 220 to 280 mg per day less in October than in the preceding month. In the case of the control sub-

jects 3 and 4, the creatinine excretion declined gradually until it reached a minimum in January, after which, on the same dietary regimen, it increased until, in May, it attained approximately the same level as in October (table 2).

These changes were not so apparent after calculation of the creatinine coefficient, which represents the quotient of the creatinine excretion expressed in milligrams, divided by the weight of the individual expressed in kilograms. Thus, if the excretion decreases or increases at the same percentage rate as the weight, then the coefficient remains unchanged. The coefficient reflects the relative changes in these 2 variables. During August and September, in the period of diet I, the coefficient increased slightly in the case of subjects 3 and 6, probably indicating that the weight decreased more rapidly than the excretion of creatinine. On the other hand, the coefficients decreased slightly in the case of the other 5 subjects; that is, the excretion decreased more rapidly than the weight. On the average, the 7 subjects excreted 2.16% less creatinine in September than in August, and the weight declined 1.13%. The differences are greater if the results from July and September are considered. During January, the fourth month of gradual decline, in the period of diet II, control subjects 3 and 4 excreted 4.7 and 6.4% less creatinine than in October, and the weight decreased only 1.4 and 2.1%, respectively (table 2).

On the whole, the downward trend of the creatinine excretion, although seemingly parallel, was at a greater rate than the loss of weight. Furthermore, the minima were not always attained during the same month. Thus, in the case of control subjects 3 and 4, the minimum weights were attained 1 and 2 months, respectively, after the minima of creatinine excretion (next to last column, table 2). In only 1 instance (subject 7), both minima occurred during the same month; however, here also the change with respect to creatinine excretion, —11.0%, was greater than the weight, —9.3%.

The rise in creatinine excretion which occurred from February to May often was accompanied by a rise in body weight.

However, the magnitude of the changes was not always equal (table 2). Although the changes with respect to weight and creatinine may have resulted from some fundamental alterations in metabolism, the effect, even though small, was not equal. In other words, the factors concerned with formation and excretion of creatinine appear to be subject to control and variation like all other biochemical phenomena. This point must be considered in any discussion concerning the creatinine coefficient.

However, in confirmation of the observations of Folin and Shaffer, it was found that the average daily excretion over a period of 1 month was to some extent related to the average weight of the individuals. Six of the subjects were muscularly well-developed, and had no evident excess fat. Subjects 2, 3, and 7 were tall and thin; subjects 4, 1, and 5 were stocky. Subject 6 was fat. The average daily creatinine excretion in September, in the order of decreasing weight (table 1, subjects 6, 3, 2, 5, 7, 4, 1) were: 2.12, 2.13, 2.14, 2.04, 1.80, 1.84, and 1.73 gm. The creatinine coefficients in the same order of subjects were: 25.5, 27.9, 29.8, 29.8, 27.1, 28.0, 27.8. On excluding the data from subject 6 who was fat, the highest coefficient was 4.9% above, and the lowest 4.6% below, the mean of 28.4 mg per kilogram per day.

The same approximate relation was observed in October, in the same order of subjects of decreasing weight as above, when diet II was given (table 2): 1.84, 1.90, 1.88, 1.78, 1.54, 1.56, 1.51 gm average creatinine excretion per day. The creatinine coefficients were: 22.2, 24.8, 26.3, 25.9, 23.2, 23.8, 24.1. Again, by excluding the first result, that of subject 6, the maximum deviations from the mean of 24.7 were -6.1 and $+6.5\%$.

Since the subjects ate the same weighed diets and, therefore, metabolized about the same quantity of protein, the differences cannot be ascribed to differences of "intensity" of exogenous protein metabolism, as postulated by Beard ('41). This is further evident from the variable and negligible

effect of the addition of casein to the basal diet of subjects 3 and 4 in November (table 2).

Effect of restricted diet II and supplementation. From weeks 12 to 28, none of the experimental subjects received supplements of vitamins or protein in the basal diet. Like that of the control subjects, the creatinine excretion decreased, but to a greater degree. The excretion dropped rapidly in the case of subjects 2, 5, and 6. Clinical symptoms appeared in varying degrees in all subjects, and were evidenced in part by the following signs: decline in work output, as compared with that of the control subjects, which became most marked during weeks 21 to 24 (figs. 1 to 4); altered cardiovascular response to various work tests; scrotal skin lesions in subjects 1, 5, 6, and 7; irritability, insomnia, lack of power of concentration which forced 3 of the subjects to give up courses of study in the evening school; pain in the legs and ankles; and increased pyruvic acid content of the blood. In the case of subjects 2, 5, and 6, the marked decrease in creatinine excretion began with, and paralleled, the development of the deficiency symptoms. However, this was not so evident in the case of subjects 1 and 7 when the results were compared with similar changes in excretion of control subjects 3 and 4. The marked changes in creatinine excretion as related to weight in the control and experimental subjects are evident in the next to last column of table 2. The results suggest that the mechanisms concerned with the production and excretion of creatinine are relatively less affected in some subjects than in others.

The supplementation of vitamins was begun in February. Because of the variability of the weekly excretion and the long time required to obtain average results, the effect of individual supplements could not be studied. The welfare of the subjects had to be considered. The creatinine excretion of subjects 1 and 2 remained stationary during February and March when only thiamine had been given, and rose slightly during the succeeding months of April and May when other supplements were given. Subjects 5, 6, and 7 received

supplementations of casein along with the vitamins, beginning in February. Relatively little change in creatinine excretion was noted in this and the succeeding month. The physical condition of subjects 6 and 7 was not apparently improved and, therefore, it was decided to give additional supplements of 100 gm lean meat, 1 glass of milk, and 1 egg per day in April. Thus, the supplemented diet of these 2 subjects contained more protein than diet I; in fact, it contained 25 gm more of meat. As was to be expected, the creatinine excretion of subjects 6 and 7 rose, and in May was —8.7 and +1.3%, respectively, of the output of October (table 2). In spite of 2 months of complete supplementation, it was still below that of September (table 1). The work output of all of the experimental subjects, although improved, was still low in May after several months of complete supplementation.

The residual effects associated with dietary restrictions are further shown by a comparison of the results from September, diet I, and June, diet III (table 1). Subjects 3 and 4, who had received casein and vitamin supplements during 7 months of the preceding period of diet II, excreted 280 and 180 mg *more* of creatinine, or +13.1 and +9.8%, respectively, in June than in September. The previous diet, therefore, did not affect the response to diet III rich in meat and other dietary essentials.

On the other hand, subjects 1 and 2, who previously had received casein for 2 months and vitamins for 4 months, excreted 40 and 260 mg *less* (—2.3 and —12.1%), respectively, than in September. Subject 5, who had previously received both supplements for 4 months, excreted 150 mg *less* (—7.4%), than in September. In contrast to this, subjects 6 and 7, who had received supplements similar to subject 5, but had also received meat, milk and eggs in the previous 2 months, excreted only 60 and 130 mg more, or +2.8 and +7.2%.

The weight of all subjects had returned to approximately the same level in June as in September. The differences in creatinine excretion, therefore, cannot be ascribed to dif-

ferences in weight. They cannot be explained entirely on the basis of the creatine or creatinine content of the diets, nor are they directly related to the protein content, which, as far as is known, is the most important source of nitrogen compounds for the synthesis of creatine and, presumably, also of creatinine. It is not likely that the differences were due to storage of creatine or creatinine because the period of 2 (subjects 1 and 2) to 4 (subjects 5, 6, and 7) months of protein supplementation before June was longer than the time usually considered necessary for nitrogen equilibrium.

Creatine determinations (by difference) were made toward the end of January, the fourth month of diet II, before supplementation was begun. The calculated excretions per day were as follows in the order of subjects 3, 4, 1, 2, 5, 6, and 7: 10, none, none, 80, 60, none, 20 mg.

DISCUSSION

The experimental conditions used in this study differed somewhat from those employed in previous studies on the factors concerned with the excretion of creatinine. All subjects received the same weighed quantities of food. The quantity of protein ingested, therefore, was the same in all subjects, regardless of weight. The restricted diet contained a considerable quantity of corn meal. The diets were administered over a long enough period to assure equilibrium and to permit observations on the variation in the excretion of creatinine. The subjects received an average of 3200 cal. per day, which was greater than had been given in most previous investigations. The diets were monotonous; each subject expected and looked for symptoms of deficiency; none of the subjects was aware of the experimental details or the results until the experiment was completed. At least once on each of 6 days each week, exercises were performed to the point of exhaustion which led to a high lactic acid content of the blood.

The factors of physical and emotional stress, which perhaps were greater in this long experiment than in previous experiments with laboratory personnel or students, should be con-

sidered in an evaluation of the results. These factors may have affected the nervous and hormonal mechanisms which exert some control not only on chemical reactions in the body tissues, but also on renal excretion. To some extent, they may have been responsible for the apparently periodic variations of the creatinine excretion which was observed in this experiment during the normal period as well as in the period of dietary restriction. However, these factors and others associated with loss of weight do not account for the gradually decreasing average rate of creatinine excretion which was observed during the period of dietary restriction, and which persisted for several months after supplementation of protein and B-complex vitamins.

Our results lend support to the theories of Folin and Shaffer mentioned in the introduction concerning the relation between the creatinine excretion and the physical and nutritional state of the individual.

SUMMARY AND CONCLUSIONS

Seven men, ages 22 to 27 years, were subjected to a uniform regimen of physical exercises and tests over a period of 50 weeks, during which the variations of creatinine excretion, body weight, and physical performance were studied. The diets were as follows: a weighed, creatinine-containing normal diet I, containing 3170 cal., 70 gm protein and adequate quantities of minerals and vitamins, during 11 weeks; a weighed, essentially creatinine-free diet II, containing 3300 cal., 45 gm protein, greatly restricted quantities of B-complex vitamins, but otherwise adequate, over a period of 36 weeks — divided into equal periods of restriction and subsequent supplementations of thiamine, riboflavin, niacin, biotin, pteroylglutamic acid, pyridoxine, pantothenic acid and 40 gm casein; approximately equal quantities of a rehabilitation diet III, containing 250 to 350 gm of meat, during the last 3 weeks. After the fifth week of diet II, all supplements were given to 2 subjects who thus served as "controls" for a period of 7 months.

Creatinine was determined in composite samples collected over 4 days each week.

The creatinine excretion varied somewhat from week to week, often in a rhythmic manner, independent of the small changes of body weight in each subject. The maximum variations of the creatinine coefficient were of the order of $\pm 5\%$ of each mean from all subjects during 9 weeks of diet I, and $\pm 3\%$ of the mean from each of the control subjects during the first 9 weeks of diet II. These variations were minimized when the monthly averages were compared; the creatinine excretion then paralleled the rise or fall of body weight. During the first 5 weeks of diet II, the average coefficients of 6 subjects, weighing 62.0 to 77.0 kg, agreed within -1.5 to $+1.6$ mg, or -6.1 to $+6.5\%$ of the mean of 24.7 mg per kilogram per day; in the case of the seventh subject, who was fat and weighed 83.1 kg, the average coefficient was 22.2 mg. The average coefficients were in the same relative order of subjects with both diets I and II.

Administration of diet II to 5 subjects resulted in gradually decreasing average creatinine coefficients and physical performance which were paralleled to some extent by development of early clinical signs and symptoms of dietary deficiency. In 2 subjects, the administration of thiamine did not increase the average creatinine excretion or the coefficient within 2 months. Complete supplementation did not immediately increase the average creatinine excretion or the coefficient, nor did it rapidly improve the physical performance. In 2 subjects, the creatinine excretion and the coefficient were not raised to that of diet I when meat, milk and 1 egg were given in addition to all supplements (a total of about 110 gm protein) during the last 2 months of diet II. The effect of the previous dietary regimen was still evident during the period of the rehabilitation diet III, both by comparison of the results with those from the control subjects and by comparison of the individual data at the end of diet period II with those of diet period I.

Therefore, dietary restrictions with respect to protein and B-complex vitamins resulting in the development of deficiency symptoms may be reflected in metabolic changes associated with the formation and excretion of creatinine. These changes may persist, and are not rapidly abolished during subsequent supplementation.

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